(12) INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(19) World Intellectual Property Organization
International Bureau

(43) International Publication Date
24 April 2003 (24.04.2003)

PCT

(10) International Publication Number
WO 03/034068 A1

(51) International Patent Classification[7]: G01N 33/574, A61K 49/16

(21) International Application Number: PCT/CA02/01556

(22) International Filing Date: 16 October 2002 (16.10.2002)

(25) Filing Language: English

(26) Publication Language: English

(30) Priority Data:
60/330,027 16 October 2001 (16.10.2001) US
PCT/CA01/01505 26 October 2001 (26.10.2001) CA

(71) Applicant *(for all designated States except US)*: **MOUNT SINAI HOSPITAL** [CA/CA]; 600 University Avenue, Toronto, Ontario M5G 1X5 (CA).

(72) Inventor; and
(75) Inventor/Applicant *(for US only)*: **DIAMANDIS, Eleftherios, P.** [CA/CA]; 44 Gerrard St. West, Suite 1504, Toronto, Ontario M5G 2K2 (CA).

(74) Agent: **BERESKIN & PARR**; 40 King Street West, 40th Floor, Toronto, Ontario M5H 3Y2 (CA).

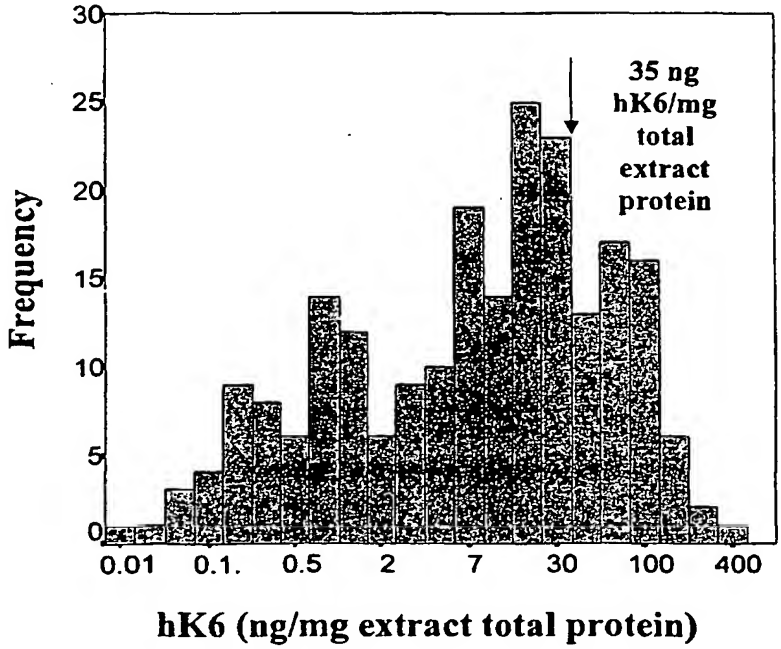
(81) Designated States *(national)*: AE, AG, AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, BZ, CA, CH, CN, CO, CR, CU, CZ, DE, DK, DM, DZ, EC, EE, ES, FI, GB, GD, GE, GH, GM, HR, HU, ID, IL, IN, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MA, MD, MG, MK, MN, MW, MX, MZ, NO, NZ, OM, PH, PL, PT, RO, RU, SD, SE, SG, SI, SK, SL, TJ, TM, TN, TR, TT, TZ, UA, UG, US, UZ, VC, VN, YU, ZA, ZM, ZW.

(84) Designated States *(regional)*: ARIPO patent (GH, GM, KE, LS, MW, MZ, SD, SL, SZ, TZ, UG, ZM, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, BG, CH, CY, CZ, DE, DK, EE, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE, SK, TR), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, GQ, GW, ML, MR, NE, SN, TD, TG).

Published:
— *with international search report*

[Continued on next page]

(54) Title: LOCALIZATION OF DISORDERS ASSOCIATED WITH KALLIKREINS



(57) Abstract: The invention relates to the localization of disorders associate d with kallikreins, more particularly ovarian cancer, by agents that have been constructed to target kallikreins, preferably kallikrein 6 and optionally additional kallikreins.

WO 03/034068 A1

For two-letter codes and other abbreviations, refer to the "Guidance Notes on Codes and Abbreviations" appearing at the beginning of each regular issue of the PCT Gazette.

TITLE: Localization of Disorders Associated with Kallikreins

FIELD OF THE INVENTION

The invention relates to the localization of disorders associated with kallikreins, in particular kalllikrein 6, by agents that have been constructed to target kallikreins.

BACKGROUND OF THE INVENTION

Until recently, the human kallikrein gene family was thought to consist of only 3 genes: pancreatic/renal kallikrein (KLK1, encoding for hK1 protein), human glandular kallikrein 2 (KLK2, encoding for hK2 protein) and human kallikrein 3 (KLK3, encoding for hK3 protein or prostate-specific antigen, PSA). The latter two kallikreins, PSA and hK2, are relatively prostate-specific and they have already found important applications as biomarkers for the diagnosis and monitoring of prostate cancer (Diamandis et al. 2000a; Diamandis 1999; McCormack et al. 1999; Chu 1997; Stenman 1999; Rittenhouse et al. 1998).

New members of the human kallikrein gene family have recently been discovered (Diamandis et al. 2000a). This gene family now consists of 15 genes which are all encoding for trypsin-like or chymotrypsin-like serine proteases, show significant homology at both the DNA and amino acid level and they are all localized at the chromosomal locus 19q13.4. This area of investigation has recently been reviewed (Diamandis et al. 2000a; Yousef and Diamandis 2001).

The KLK6 gene (encoding for human kallikrein 6, hK6) has been cloned independently by three groups of investigators and was previously given the names zyme (Little et al. 1997) [cloned from brain tissue], protease M (Anisowicz et al. 1996) [cloned from breast tissue] and neurosin (Yamashiro et al. 1997) [cloned from a colon carcinoma cell line]. Recently, uniform nomenclature for all newly discovered and the traditional kallikrein genes has been established (Diamandis et al. 2000b). The KLK6 gene encodes for a trypsin-like serine protease of 244 amino acids in length, of which 16 amino acids constitute the signal peptide and 5 amino acids, the activation peptide. The mature enzyme consists of 223 amino acids. It has been previously predicted that hK6 is a secreted protein (Little et al. 1997; Anisowicz et al. 1996; Yamashiro et al. 1997; Yousef et al. 1999). This was recently verified by finding hK6 protein in various biological fluids, including cerebrospinal fluid, breast milk, nipple aspirate fluid, breast cyst fluid, male and female serum, seminal plasma, amniotic fluid and breast cancer cytosols (Diamandis et al. 2000c). Little et al. suggested that this enzyme has amyloidogenic potential in the brain and may play a role in the development and progression of Alzheimer's disease (Little et al. 1997). Anisowicz et al. have cloned the same gene by the method of differential display, and found that it is down-regulated in aggressive forms of breast cancer (Anisowicz et al. 1996). The same gene was cloned by Yamashiro et al. from the human colon adenocarcinoma cell line COLO 201 (Yamashiro et al. 1997).

The genomic organization and the hormonal regulation of the KLK6 gene has been investigated and its tissue expression by reverse-transcription-polymerase chain-reaction has been studied (Yousef et al. 1999). The gene is abundantly expressed in many tissues, including the salivary gland, brain, uterus, heart, thymus, prostate, liver, breast, thyroid, spleen, placenta, trachea, testis and kidney (Yousef et al. 1999).

SUMMARY OF THE INVENTION

The invention relates to a method for detecting and locating disorders associated with one or more,

preferably two or more, kallikreins.

The invention contemplates compositions and methods for imaging tumor cells and tissues *in vivo* and *in situ*, which can be particularly useful for imaging abnormal tissue and organs, including sites of primary and metastatic tumors.

In accordance with an aspect of the invention the disorder is associated with kallikrein 6 (hK6) and may additionally include one or more of kallikrein 4 (hK4), kallikrein 5 (hK5), kallikrein 7 (hK7), kallikrein 8 (hK8), kallikrein 9 (hK9), kallikrein 10 (hK10), kallikrein 11 (hK11), kallikrein 12 (hK12), kallikrein 13 (hK13), kallikrein 14 (hK14), and kallikrein 15 (hK15). In a preferred embodiment the disorder is cancer, more preferably ovarian cancer, most preferably epithelial ovarian cancer.

The invention includes a method of identifying the presence of a tumor tissue bearing a kallikrein in an excised tissue sample from a subject (e.g. biopsy sample) comprising the steps of: exposing the tissue to an amount of a detectably-labeled kallikrein binding molecule or agent effective to bind to the kallikrein; and examining the sample for the presence or absence of bound labeled kallikrein.

The invention also provides a method of evaluating an excised mammalian tissue sample for the presence of a tumor bearing a kallikrein (e.g. ovarian tumor) comprising the steps of: exposing the tissue to an amount of a detectably labeled kallikrein binding molecule or agent effective to bind to the kallikrein tumor tissue, and examining the sample for the presence or absence of labeled kallikrein.

In accordance with an aspect of the invention an *in vivo* method is provided comprising administering to a subject an agent that has been constructed to target one or more kallikreins. The invention contemplates an *in vivo* method comprising administering to a mammal one or more agent that carries a label for imaging and binds to a kallikrein, preferably hK6, and then imaging the mammal..

In an embodiment, the invention provides a method of imaging a tumor, preferably an ovarian tumor in a subject, the tumor having one or more kallikreins selected from the group consisting of hK4, hK5, hK6, hK7, hK8, hK9, hK10, hK11, hK12, hK13, hK14, and hK15, comprising the steps of: delivering into the subject an amount of a detectably-labeled kallikrein binding molecule effective to image the tumor; and scanning the subject to determine the distribution of the labeled kallikrein. In an embodiment the tumor is an ovarian tumor having hK5 and hK6; hK4, hK5, hK6, hK7, and hK8; or, hK4, hK5, hK6, hK7, hK8, hK9, hK10, and hK11.

According to a preferred aspect of the invention, an *in vivo* method for imaging ovarian cancer is provided comprising:

(a) injecting a patient with an agent that binds to kallikrein 6 the agent carrying a label for imaging the ovarian cancer;

(b) allowing the agent to incubate *in vivo* and bind to kallikrein 6 associated with the ovarian cancer; and

(c) detecting the presence of the label localized to the ovarian cancer.

The invention also contemplates the methods described herein using multiple markers for ovarian cancer. For example, a method for imaging ovarian cancer may further comprise in step (a) above, injecting the patient with one or more of an agent that binds to human stratum corneum chymotryptic enzyme (HSCCE, also known as kallikrein 7 or hK7), kallikrein 4, kallikrein 5, kallikrein 8, kallikrein 9, kallikrein 10, kallikrein 11, kallikrein 12, kallikrein 13, kallikrein 14, kallikrein 15, CA125, CA15-3, CA19-9, CA72-4, OVX1, lysophosphatidic acid

(LPA) or carcinoembryonic antigen (CEA).

In an aspect, a composition adapted for imaging ovarian cancer comprising an agent that binds to one or more of hK4, hK5, hK6, hK7, hK8, hK9, hK10, hK11, hK12, hK13, hK14, and hK15, and a label for imaging is provided to a patient or to a tissue sample, and the kallikrein is located in the patient or sample by visualizing the imaging agent bound to the kallikrein. In an embodiment, the composition comprises an agent that binds to hK6 and a label for imaging, and an agent that binds to one or more of hK4, hK5, hK7, hK8, hK9, hK10, and hK11, wherein the agent for hK4, hK5, hK7, hK8, hK9, hK10, and hK11 is labeled so that each agent is distinguished during the imaging. In another embodiment, the composition comprises agents that bind to hK5 and hK6, or hK4, hK5, hK6, hK7, and hK8.

The invention also provides a nucleic acid encoding a chimeric polypeptide comprising a substance that binds to a kallikrein (e.g. hK6) and a label for imaging the kallikrein. The invention also provides an expression vector comprising a nucleic acid encoding such a chimeric polypeptide. The invention provides a cell e.g. a transformed viral-infected cell, comprising a nucleic acid encoding a chimeric polypeptide of the invention. The cell can be a bacterial, a yeast, an insect, or a mammalian cell. The invention provides a recombinant chimeric polypeptide produced by such a cell.

The invention still further provides a pharmaceutical formulation comprising a composition comprising an agent that binds a kallikrein (e.g. anti-hK6) and a label for imaging a tumor, preferably an ovarian tumor, and a pharmaceutically acceptable excipient, and wherein the composition is present in an amount sufficient to enhance a computer assisted tomography (CAT) image, a magnetic resonance spectroscopy (MRS) image, a magnetic resonance imaging (MRI) image, a positron emission tomography (PET) image, a single-photon emision computed tomography (SPECT) image, or a bioluminescence image (BLI), or equivalents thereof, when the pharmaceutical formulation is administered to an individual, or applied to a tissue or organ *in situ*, in a sufficient amount.

The invention contemplates a method for *in situ* or *in vivo* imaging of a cell, tissue, an organ, or a full body comprising administering a pharmaceutical formulation of the invention, in an amount sufficient to enhance an image, wherein the image is generated by computer assisted tomography (CAT), magnetic resonance spectroscopy (MRS) image, magnetic resonance imaging (MRI), positron emission tomography (PET), single-photon emision computed tomography (SPECT), or bioluminescence imaging (BLI), or equivalents thereof.

The invention also provides a method of *in situ* or *in vivo* imaging of a cell, a tissue, an organ or a full body comprising the following steps: (a) providing a pharmaceutical formulation of the invention; (b) providing an imaging device, wherein the imaging device is computer assisted tomography (PET), magnetic resonance spectroscopy (MRS), magnetic resonance imaging (MRI), a positron emission tomography (PET), single-photon emision computed tomography (SPECT), or bioluminescence imaging (BLI) or equivalent; (c) administering the pharmaceutical formulation in an amount sufficient to generate the cell, tissue, or body image; and (d) imaging the distribution of the pharmaceutical formulation of step (a) with the imaging device, thereby imaging the cell, tissue or body.

In one aspect, the pharmaceutical formulation is administered to a human, such as a cancer patient, in particular an ovarian cancer patient, or a patient suspected of having or being screened for cancer, in particular ovarian cancer. In an embodiment, the pharmaceutical formulation is administered intravenously. The image may be taken between 2 minutes and 24 hours after administration of the pharmaceutical formulation.

The invention contemplates a method for *in vivo* imaging tumor neovasculature in a subject comprising: (a) providing a pharmaceutical formulation of the invention; (b) providing an imaging device, wherein the imaging device is computer assisted tomography (CAT), magnetic resonance spectroscopy (MRS), magnetic resonance imaging (MRI), positron emission tomography (PET), single-photon emission computed tomography (SPECT), bioluminescence imaging (BLI) or equivalent; (c) administering the pharmaceutical formulation in an amount sufficient to image the tumor neovasculature; and, (d) imaging the distribution of the pharmaceutical formulation of step (a) with the imaging device, thereby imaging the tumor neovasculature.

The invention provides a method for *in situ* or *in vivo* screening for an anti-tumor agent by imaging tumor neovasculature in an individual comprising the following steps: (a) providing a composition comprising a chimeric polypeptide or a pharmaceutical formulation of the invention, and a test compound; (b) providing an imaging device, wherein the imaging device is computer assisted tomography (CAT), magnetic resonance spectroscopy (MRS), magnetic resonance imaging (MRI), positron emission tomography (PET), single-photon emission computed tomography (SPECT), bioluminescence imaging (BLI) or equivalent; (c) administering the composition of step (a) in an amount sufficient to image the tumor and imaging the distribution of the composition with the imaging device, thereby imaging the tumor; (d) administering the test compound; and, (e) imaging the distribution of the composition with the imaging device, thereby imaging the tumor, wherein a decrease in the amount of tumor neovasculature indicates that the compound is an anti-tumor or an anti-angiogenic agent.

In certain embodiments of the methods and compositions of the invention, the agent or kallikrein binding molecule is an antibody that recognizes or binds the kallikrein. In other embodiments of the invention the agent is a chemical entity which recognizes or binds the kallikrein.

The agent or kallikrein binding molecule carries a label to image the kallikreins. Examples of labels useful for imaging in accordance with the present invention are radiolabels, fluorescent labels (e.g fluorescein and rhodamine), nuclear magnetic resonance active labels, positron emitting isotopes detectable by a positron emission tomography ("PET") scanner, chemiluminescers such as luciferin, bioluminescent labels, and enzymatic markers such as peroxidase or phosphatase. Short-range radiation emitters, such as isotopes detectable by short-range detector probes can also be employed.

The invention also relates to kits for carrying out the methods of the invention. In an embodiment the invention contemplates kits comprising the pharmaceutical formulations, chimeric polypeptides, or nucleic acids encoding the chimeric polypeptides of the invention.

Other objects, features and advantages of the present invention will become apparent from the following detailed description. It should be understood, however, that the detailed description and the specific examples while indicating preferred embodiments of the invention are given by way of illustration only, since various changes and modifications within the spirit and scope of the invention will become apparent to those skilled in the art from this detailed description.

DESCRIPTION OF THE DRAWINGS

The invention will now be described in relation to the drawings in which:

Figure 1(A) is a graph showing the frequency distribution of hK6 specific activity in ovarian tumor extracts. The value of 35ng/mg of total protein corresponds to the limit that, according to Chi square analysis, gives the best prediction of overall survival of the study population. See Figure 1(B) for Chi square plot. Tumors with

hK6 in excess of 35 ng/mg total protein were classified as hK6 positive and those with values less than or equal to 35 ng/mg total protein were classified as hK6 negative. 30% of the tumors were classified as positive by this criterion. (B) Plot of hK6 tumor specific activity versus Chi-square statistic to determine the limit between hK6 positive and hK6 negative tumors that is most predictive of overall survival. Maximum predictive potential occurred between 28 to 40 ng hK6 total extract protein with a peak at 35 ng hK6/mg total extract protein.

Figure 2 is a graph showing a comparison of hK6 concentration in extracts from normal ovarian tissues ("normal"), and ovarian cancer ("cancer"). N indicates the number of specimens in each group. Horizontal bars represent the median hK6 specific activity (ng hK6/mg total extract protein) in each group. The Krustal Wallis test showed that extracted hK6 specific activity was significantly elevated in the ovarian tumor preparations ($P<0.001$).

Figure 3 is a graph showing the distribution of hK6 specific activity (ng hK6/mg total protein) in tumor extracts from stage I/II and stage III/IV ovarian cancer patients. N indicates the number of tumors comprising each group. Horizontal bars represent the median value of hK6 tumor specific activity. The Mann-Whitney test demonstrated that hK6 specific activity was significantly elevated in tumors from patients with stage III/IV ovarian cancer ($P=0.002$).

Figure 4 shows Kaplan-Meier survival curves of the entire patient population under study:effect of hK6 status. Top: progression-free survival (PFS). Bottom: overall survival (OS). The patient number in each group (n) is indicated as is the statistical significance (P value) of the survival difference between hK6 positive and hK6 negative groups. The adverse effect of hK6 positivity on both time to progression and overall survival was significant.

Figure 5 are graphs showing the effect of hK6 status (positive or negative) on progression –free survival (PFS) and on overall survival (OS) among patients with Grade I and II ovarian tumor. The patient number in each group (n) is indicated as is the statistical significance (P value) of the survival difference between hK6 positive and hK6 negative individuals. The adverse effect of hK6 positivity both on time to progression and on overall survival was significant ($P\leq 0.002$).

Figure 6 is a blot showing immunohistochemical localization of hK6 in ovarian neoplasms of varying malignant potential, cell type, and origin (epithelial versus mesenchymal). (A) Invasive papillary serous adenocarcinoma, the common malignant epithelial tumor of the ovary. Note strong cytoplasmic staining of many tumor cells, and absence of any staining of stroma or vessels. (B) Serous cystadenofibroma, a benign, mixed epithelial and fibrous neoplasm. Innumostaining is absent in the fibrous component, but strongly positive in the cytoplasm of the epithelium lining the cysts. (C) Ovarian leiomyoma, a benign smooth muscle tumor. Note the absence of staining. (D) Mucinous epithelial tumor of low malignant potential, an epithelial tumor of intermediate grade. Note weak, diffuse cytoplasmic staining of neoplastic epithelium and absent staining in supportive stroma (far left).

DETAILED DESCRIPTION OF THE INVENTION

The invention provides a method for imaging tumors associated with one or more kallikreins, preferably kallikreins associated with ovarian cancer, most preferably hK6, and optionally hK4, hK5, hK7, hK8, hK9, hK10, hK11, hK12, hK13, hK14, and hK15.

The invention also contemplates the methods described herein using multiple markers for ovarian cancer. For example, a method for imaging ovarian cancer may further comprise providing to a patient or a tissue sample

one or more of an agent that binds to human stratum corneum chymotryptic enzyme (HSCCE), kallikrein 4, kallikrein 5, kallikrein 8, kallikrein 9, kallikrein 10, kallikrein 11, CA125, CA15-3, CA19-9, OVX1, lysophosphatidic acid (LPA), or carcinoembryonic antigen (CEA), preferably hK4, hK5, hK6, hK7, and hK8. Preferably each agent is labeled so that each marker can be distinguished during the imaging.

The term "hK6"or "kallikrein 6" refers to human kallikrein 6, (also known as zyme, protease M, and neurosin) a trypsin-like serine protease of 244 amino acids in length, of which 16 amino acids constitute the signal peptide and 5 amino acids, the activation peptide (7, 8, and 9). The amino acid sequence for hK6 can be found at GenBank Accession Nos. AF013988, AF149289, HSU62801, D78203, and NM002774. The terms " kallikrein 4" or "hK4", "kallikrein 5" or "hK5", "kallikrein 7" or "hK7", "kallikrein 8" or "hK8", "kallikrein 9" or "hK9", "kallikrein 10" or "hK10", "kallikrein 11" or "hK11", "kallikrein 12" or "hK12", "kallikrein 13" or "hK13", "kallikrein 14" or "hK14", and "kallikrein 15" or "hK15" refer to human kallikrein proteins that have the sequences provided in GenBank and identified by the Accession Nos. in Table 6. The kallikrein proteins are also described in the publications referenced in the GenBank records.

The kallikein proteins referred to herein include all homologs, naturally occurring allelic variants, isoforms and precursors of the kallikrein sequences set out in the GenBank records referred to in Table 6. In general for example, naturally occurring allelic variants of a human kallikrein protein will share significant homology (70-90%) to the sequences shown in the GenBank Accession Nos.. Allelic variants may contain conservative amino acid substitutions from the kallikrein sequences or will contain a substitution of an amino acid from a corresponding position in a kallikrein homologue such as, for example, the murine kallikrien homologue.

In an aspect of the invention tissue samples are imaged using methods of the invention. Tissue samples can be excised from a subject and treated with one or more agent that binds to a kallikrein and carries a label for imaging the kallikrein. A kallikrein is located in the sample by visualizing the label bound to kallikrein in the sample. The tissue sample may be a tumor tissue, or a tissue suspected of comprising tumor tissue or cells, in particular ovarian tumor tissue or cells.

In an embodiment, an immunohistology method is used to assay for the presence or absence of a kallikrein in a tissue sample such as an ovarian tumor tissue sample. The tissue sample may be treated with antibodies specific for a kallikrein and antibodies bound to the kallikrein can be detected by treating with a biotinylated antibody against the kallikrein specific antibody followed by treatment with an enzyme tagged avidin (e.g. avidin-peroxidase) and enzyme substrate (e.g. chromogenic peroxidase substrate).

The invention also provides *in vivo* methods whereby a subject or patient is administered one or more agents that carry an imaging label, and that are capable of targeting or binding to a kallikrein. The agent is allowed to incubate *in vivo* and bind to the kallikrein(s) associated with a tumor, preferably ovarian tumors. The presence of the label is localized to the ovarian cancer, and the localized label is detected using imaging devices known to those skilled in the art.

The term "subject" or "patient" refers to a warm-blooded animal such as a mammal that is afflicted, suspected of being afflicted with, or being screened for a condition associated with a kallikrein, in particular cancer, more particularly ovarian cancer. Preferably, "subject" refers to a human.

The agent may be an antibody or chemical entity that recognizes or binds to the kallikrein(s). The term "antibody" includes both intact antibodies having at least two heavy (H) chains and two light (L) chains inter-

connected by disulfide bonds and antigen binding fragments thereof, or equivalents thereof, either isolated from natural sources, recombinantly generated or partially or entirely synthetic. Examples of antigen binding fragments include, e.g., Fab fragments, $F(ab')_2$ fragments, Fd fragments, dAb fragments, isolated complementarity determining regions (CDR), single chain antibodies, chimeric antibodies, humanized antibodies, human antibodies made in non-human animals (e.g., transgenic mice) or any form of antigen binding fragment.

Antibodies including monoclonal and polyclonal antibodies, fragments and chimeras, may be prepared using methods known to those skilled in the art. Preferably, antibodies used in the methods of the invention are reactive against a kallikrein if they bind with a K_a of greater than or equal to 10^{-7} M.

An agent may be a peptide that mimics the epitope for an antibody specific for a kallikrein and binds to the kallikrein. The peptide may be produced on a commercial synthesizer using conventional solid phase chemistry. By way of example, a peptide may be prepared that includes either tyrosine lysine, or phenylalanine to which N_2S_2 chelate is complexed (See U.S. Patent No. 4,897,255). The anti-kallikrein peptide conjugate is then combined with a radiolabel (e.g. sodium ^{99m}Tc pertechnetate or sodium ^{188}Re perrhenate) and it may be used to locate a kallilkrein producing tumor.

The agent carries a label to image the kallikreins. The agent may be labelled for use in radionuclide imaging. In particular, the agent may be directly or indirectly labelled with a radioisotope. Examples of radioisotopes that may be used in the present invention are the following: ^{277}Ac, ^{211}At, ^{128}Ba, ^{131}Ba, ^{7}Be, ^{204}Bi, ^{205}Bi, ^{206}Bi, ^{76}Br, ^{77}Br, ^{82}Br, ^{109}Cd, ^{47}Ca, ^{11}C, ^{14}C, ^{36}Cl, ^{48}Cr, ^{51}Cr, ^{62}Cu, ^{64}Cu, ^{67}Cu, ^{165}Dy, ^{155}Eu, ^{18}F, ^{153}Gd, ^{66}Ga, ^{67}Ga, ^{68}Ga, ^{72}Ga, ^{198}Au, ^{3}H, ^{166}Ho, ^{111}In, ^{113m}In, ^{115m}In, ^{123}I, ^{125}I, ^{131}I, ^{189}Ir, ^{191m}Ir, ^{192}Ir, ^{194}Ir, ^{52}Fe, ^{55}Fe, ^{59}Fe, ^{177}Lu, ^{15}O, $^{191m-191}$Os, ^{109}Pd, ^{32}P, ^{33}P, ^{42}K, ^{226}Ra, ^{186}Re, ^{188}Re, ^{82m}Rb, ^{153}Sm, ^{46}Sc, ^{47}Sc, ^{72}Se, ^{75}Se, ^{105}Ag, ^{22}Na, ^{24}Na, ^{89}Sr, ^{35}S, ^{38}S, ^{177}Ta, ^{96}Tc, ^{99m}Tc, ^{201}Tl, ^{202}Tl, ^{113}Sn, ^{117m}Sn, ^{121}Sn, ^{166}Yb, ^{169}Yb, ^{175}Yb, ^{88}Y, ^{90}Y, ^{62}Zn and ^{65}Zn. Preferably the radioisotope is ^{131}I, ^{125}I, ^{123}I, ^{111}I, ^{99m}Tc, ^{90}Y, ^{186}Re, ^{188}Re, ^{32}P, ^{153}Sm, ^{67}Ga, ^{201}Tl ^{77}Br, or ^{18}F, and is imaged with a photoscanning device.

Procedures for labeling biological agents with radioactive isotopes are generally known in the art. U.S. Pat. No. 4,302,438 describes tritium labeling procedures. Procedures for iodinating, tritium labeling, and ^{35}S labeling especially adapted for murine monoclonal antibodies are described by Goding, J. W. (supra, pp 124-126) and the references cited therein. Other procedures for iodinating biological agents, such as antibodies, binding portions thereof, probes, or ligands, are described in the scientific literature (see Hunter and Greenwood, Nature 144:945 (1962), David et al., Biochemistry 13:1014-1021 (1974), and U.S. Pat. Nos. 3,867,517 and 4,376,110). Iodinating procedures for agents are described by Greenwood, F. et al., Biochem. J. 89:114-123 (1963); Marchalonis, J., Biochem. J. 113:299-305 (1969); and Morrison, M. et al., Immunochemistry, 289-297 (1971). ^{99m}Tc-labeling procedures are described by Rhodes, B. et al. in Burchiel, S. et al. (eds.), Tumor Imaging: The Radioimmunochemical Detection of Cancer, New York: Masson 111-123 (1982) and the references cited therein. Labelling of antibodies or fragments with technetium-99m are also described for example in U.S. Pat. No. 5,317,091, U.S. Pat. No. 4,478,815, U.S. Pat. No. 4,478,818, U.S. Pat. No. 4,472,371, U.S. Pat. No. Re 32,417, and U.S. Pat. No. 4,311,688. Procedures suitable for ^{111}In-labeling biological agents are described by Hnatowich, D. J. et al., J. Immul. Methods, 65:147-157 (1983), Hnatowich, D. et al., J. Applied Radiation, 35:554-557 (1984), and Buckley, R. G. et al., F.E.B.S. 166:202-204 (1984).

In the case of a radiolabeled agent, the agent may be administered to the patient, it is localized to the

tumor having a kallikrein with which the agent binds, and is detected or "imaged" *in vivo* using known techniques such as radionuclear scanning using e.g., a gamma camera or emission tomography. [See for example A. R. Bradwell et al., "Developments in Antibody Imaging", Monoclonal Antibodies for Cancer Detection and Therapy, R. W. Baldwin et al., (eds.), pp. 65-85 (Academic Press 1985)]. A positron emission transaxial tomography scanner can also be used where the radiolabel emits positrons (e.g., ^{11}C, ^{18}F, ^{15}O, and ^{13}N).

Whole body imaging techniques using radioisotope labeled agents can be used for locating both primary tumors and tumors which have metastasized. Antibodies specific for kallikreins, or fragments thereof having the same epitope specificity, are bound to a suitable radioisotope, or a combination thereof, and administered parenterally. For ovarian cancer, administration preferably is intravenous. The bio-distribution of the label can be monitored by scintigraphy, and accumulations of the label are related to the presence of ovarian cancer cells. Whole body imaging techniques are described in U.S. Pat. Nos. 4,036,945 and 4,311,688. Other examples of agents useful for diagnosis and therapeutic use which can be coupled to antibodies and antibody fragments include metallothionein and fragments (see, U.S. Pat. No. 4,732,864). These agents are useful in diagnosis staging and visualization of cancer, in particular ovarian cancer, so that surgical and/or radiation treatment protocols can be used more efficiently.

The agent may carry a bioluminescent or chemiluminescent label. Such labels include polypeptides known to be fluorescent, bioluminescent or chemiluminescent, or, that act as enzymes on a specific substrate (reagent), or can generate a fluorescent, bioluminescent or chemiluminescent molecule. Examples of bioluminescent or chemiluminescent labels include luciferases, aequorin, obelin, mnemiopsin, berovin, a phenanthridinium ester, and variations thereof and combinations thereof. The pharmaceutical formulation of the invention can further comprise a substrate for the bioluminescent or chemiluminescent polypeptide. For example, the chemiluminescent polypeptide can be luciferase and the reagent luciferin. The substrate can be administered before, at the same time (e.g., in the same formulation), or after administration of the chimeric polypeptide (including the enzyme).

A substrate for a bioluminescent or chemiluminescent label can be administered before, at the same time (e.g., in the same formulation), or after administration of the agent. Thus, the invention contemplates a pharmaceutical formulation comprising an agent labeled with a bioluminescent or chemiluminescent label and further comprising a substrate for the bioluminescent or chemiluminescent polypeptide.

An agent may comprise a paramagnetic compound, such as a polypeptide chelated to a metal, e.g., a metalloporphyrin. The paramagnetic compound may also comprise a monocrystalline nanoparticle, e.g., a nanoparticle comprising a lanthanide (e.g., Gd) or iron oxide; or, a metal ion comprising a lanthanide. "Lanthanides" refers to elements of atomic numbers 58 to 70, a transition metal of atomic numbers 21 to 29, 42 or 44, a Gd(III), a Mn(II), or an element comprising an Fe element. Paramagnetic compounds can also comprise a neodymium iron oxide ($NdFeO_3$) or a dysprosium iron oxide ($DyFeO_3$). Examples of elements that are useful in magnetic resonance imaging include gadolinium, terbium, tin, iron, or isotopes thereof. (See, for example, Schaefer et al., (1989) JACC 14, 472-480; Shreve et al., (1986) Magn. Reson. Med. 3, 336-340; Wolf, G L., (1984) Physiol. Chem. Phys. Med. NMR 16, 93-95; Wesbey et al., (1984) Physiol. Chem. Phys. Med. NMR 16, 145-155; Runge et al., (1984) Invest. Radiol. 19, 408-415 for discussions on *in vivo* nuclear magnetic resonance imaging.)

An image can be generated in a method of the invention by computer assisted tomography (CAT), magnetic resonance spectroscopy (MRS) image, magnetic resonance imaging (MRI), positron emission tomography (PET), single-photon emision computed tomography (SPECT), or bioluminescence imaging (BLI) or equivalent.

Computer assisted tomography (CAT) and computerized axial tomography (CAT) systems and devices well known in the art can be utilized in the practice of the present invention. (See, for example, U.S. Patent Nos. 6,151,377; 5,946,371; 5,446,799; 5,406,479; 5,208,581; 5,109,397). The invention may also utilize animal imaging modalities, such as MicroCAT.TM. (ImTek, Inc.).

Magnetic resonance imaging (MRI) systems and devices well known in the art can be utilized in the practice of the present invention. In magnetic resonance methods and devices, a static magnetic field is applied to a tissue or a body in order to define an equilibrium axis of magnetic alignment in a region of interest. A radio frequency field is then applied to the region in a direction orthogonal to the static magnetic field direction to excite magnetic resonance in the region. The resulting radio frequency signals are then detected and processed, and the exciting radio frequency field is applied. The resulting signals are detected by radio-frequency coils that are placed adjacent to the tissue or area of the body of interest. (For a description of MRI methods and devices see, for example, U.S. Patent Nos. 6,151,377; 6,144,202; 6,128,522; 6,127,825; 6,121,775; 6,119,032; 6,115,446; 6,111,410; 602,891; 5,555,251; 5,455,512; 5,450,010; 5,378,987; 5,214,382; 5,031,624; 5,207,222; 4,985,678; 4,906,931; 4,558,279). MRI and supporting devices are commercially available for example, from Bruker Medical GMBH; Caprius; Esaote Biomedica; Fonar; GE Medical Systems (GEMS); Hitachi Medical Systems America; Intermagnetics General Corporation; Lunar Corp.; MagneVu; Marconi Medicals; Philips Medical Systems; Shimadzu; Siemens; Toshiba America Medical Systems; including imaging systems, by, e.g., Silicon Graphics. The invention may also utilize animal imaging modalities such as micro-MRIs.

Positron emission tomography imaging (PET) systems and devices well known in the art can be utilized in the practice of the present invention. For example, a method of the invention may use the system designated Pet VI located at Brookhaven National Laboratory. For descriptions of PET systems and devices see, for example, U.S. Pat. Nos. 6,151,377; 6,072,177; 5,900,636; 5,608,221; 5,532,489; 5,272,343; 5,103,098. Animal imaging modalities such as micro-PETs (Corcorde Microsystems, Inc.) can also be used in the invention.

Single-photon emission computed tomography (SPECT) systems and devices well known in the art can be utilized in the practice of the present invention. (See, for example, U.S. Patents. Nos. 6,115,446; 6,072,177; 5,608,221; 5,600,145; 5,210,421; 5,103,098.) The methods of the invention may also utilize animal imaging modalities, such as micro-SPECTs.

Bioluminescence imaging includes bioluminescence, fluorescence or chemiluminescence or other photon detection systems and devices that are capable of detecting bioluminescence, fluorescence or chemiluminescence. Sensitive photon detection systems can be used to detect bioluminescent and fluorescent proteins externally; see, for example, Contag (2000) Neoplasia 2:41-52; Zhang (1994) Clin. Exp. Metastasis 12:87-92. The methods of the invention can be practiced using any such photon detection device, or variation or equivalent thereof, or in conjunction with any known photon detection methodology, including visual imaging. By way of example, an intensified charge-coupled device (ICCD) camera coupled to an image processor may be used in the present invention. (See, e.g., U.S. Pat. No. 5,650,135). Photon detection devices are also commercially available from

Xenogen, Hamamatsue.

The invention also contemplates kits for carrying out the methods of the invention. The kits include an antibody or an antibody fragment which binds specifically to an epitope of a kallikrein, and means for detecting binding of the antibody to its epitope associated with tumor cells, either as concentrates (including lyophilized compositions), which may be further diluted prior to use or at the concentration of use, where the vials may include one or more dosages. Where the kits are intended for *in vivo* use, single dosages may be provided in sterilized containers, having the desired amount and concentration of agents. Containers that provide a formulation for direct use, usually do not require other reagents, as for example, where the kit contains a radiolabelled antibody preparation for *in vivo* imaging.

The kits may also comprise instructional material teaching methodologies, e.g., how and when to administer the pharmaceutical compositions, how to apply the compositions and methods of the invention to imaging systems, e.g., computer assisted tomography (CAT), magnetic resonance spectroscopy (MRS), magnetic resonance imaging (MRI), positron emission tomography (PET), single-photon emission computed tomography (SPECT) or bioluminescence imaging (BLI). Kits containing pharmaceutical compositions (e.g., chimeric polypeptides, vectors, nucleic acids) may also include directions as to indications, dosages, routes and methods of administration, and the like.

The following non-limiting examples are illustrative of the present invention:

Example 1

KLK6 Human Normal Tissue

MATERIALS AND METHOD

This is an immunohistochemical study on almost all normal human tissues, in order to establish the expression of hK6. Parts of an organ with different histology (e.g. stomach, fundus, body, antrum) were examined separately. A paraffin block of three different cases for every tissue (organ- all parts with different histology) was selected. Cases with malignancy in adjacent sites of the organ were excluded, in order to avoid phenotypic changes that may be associated with cancer. Tissues that exist in several organs (e.g. fat, muscle, vessels, peripheral nerves, ganglia, and neuroendocrine cells) were not studied separately. A total of 199 paraffin blocks were examined. 165 blocks were from archival or current material from 132 cases and the rest were autopsy material from two cases (Table 1).

The immunohistochemical method of avidin-biotin complex was performed on 4μm thick sections, using two hK6-specific antibodies, one rabbit polyclonal and one mouse monoclonal. Both antibodies were raised in-house against full length hK6, produced recombinantly in a mammalian stable cell line system (Little et al. 1997). The recombinant hK6 protein was purified by HPLC, as previously described (Little et al. 1997). The specificity of the antibodies was evaluated during development of the immunofluorometric assay, as previously described and by Western blot analysis (Diamandis et al. 2000c). No detectable cross-reactivity was found from other closely related antigens, like hK2, hK3 and hK10 (Diamandis et al. 2000c). All tissues were fixed in buffered-formalin. Staining procedures included deparaffinization in xylene and then two changes of xylene at room temperature, followed by rehydration by transfer through graded alcohols. Endogenous peroxidase activity was blocked with 0.5% H_2O_2 in methanol for 10 min. The sections were pretreated with citrate buffer (pH 6.1) in microwave for 5 min and incubated overnight with the hK6 primary rabbit polyclonal antibody (1:500) and the hK6 primary mouse

monoclonal antibody (1:150) in 3% BSA. After washing with TRIS-buffered saline (TBS; pH=7.6) for 15 min, they were incubated with a biotinylated secondary antibody for 10 min. After washing with TBS, we incubated with the avidin peroxidase complex for 10 min. The next steps included application of diaminobenzidine (DAB) substrate and incubation for 10 min, washing in running water, staining with hematoxylin for 1 min, washing in running water again, dehydration through graded alcohols and covering by DPX. A negative control section was always included in which the primary antibody was substituted with non-immune rabbit or mouse serum. Also, for selected tissues, immunoabsorbtion of the primary hK6 antibody was performed by mixing it for 1h with excess recombinant hK6, before immunostaining.

The staining pattern, the distribution of the immunostaining in each tissue and the intensity of the staining were studied in detail.

RESULTS

The hK6 immunoreactivity using the polyclonal and the monoclonal antibody was generally localized in the cytoplasm. Both antibodies revealed the same distribution of the antigen in all tissues. Replacement of the primary antibody with non-immune serum or immunoabsorbtion of the primary antibody abolished the immunostaining in all examined tissues. These findings support that the staining procedure is specific for hK6. The protein was revealed in a variety of tissues, indicating that this is not a tissue specific protein. hK6 is mainly expressed by glandular tissues but the data suggested that it could also serve as a neuroendocrine marker. The distribution and the expression levels of hK6 in various tissues are described below and further summarized in Table 2.

Central and peripheral nervous system

A strong and diffuse positivity in the epithelium of the choroid plexus was observed. In the cerebellum the antigen was expressed weakly in the Purkinje cells and in the stellate (basket cells), while the granular cells were negative. In the whole central nervous system the nerve cells showed a weak immunostaining as well. Glial cells showed a weaker expression focally. The staining of the peripheral nerves was intense.

Female reproductive system

The epithelium of the breast stained positive. Cytoplasmic and brush border distribution was observed and luminal secretions were positive as well. A similar immunophenotype appeared in the columnar epithelium of the endometrium and the endocervix of the uterus. A characteristic droplet-like, widely distributed expression was noticed in the endometrium, but staining in the endocervix was focal and paranuclear. No major differences were seen during the menstrual cycle. The myometrium was negative. A weak and focal expression by the squamous cervical epithelium could not be safely considered as positive. The squamous epithelium of the vagina was negative. A supranuclear cytoplasmic, brush border and cilia staining was revealed in the fallopian tubes. Characteristic was the positivity in the premordial follicles of the ovary. In the placenta the protein was localized in the endothelia, in calcifications of the villi, as well as in "X" cells and focally, in trophoblastic cells.

Genitourinary tract

A strong diffuse immunostaining in the prostate columnar cells was observed. Basal cells remained unstained. As a double-staining method was not used, it was not possible to evaluate the positivity of the neuroendocrine cells. Generally, hK6 had the same immunostaining pattern in the benign prostate gland, as hK2 and hK3. A focal, mainly suprabasal, cytoplasmic, brush border and cilia staining in the epithelium of the

epididymis, seminal vesicles and spermatic duct was the rule. The urinary tubuli of the kidney showed an immunoexpression as well. It is doubtful if a weak focal expression by the umbrella cells of the urothelium and the Leydig cells of the testis could be considered as positive. Cavernous bodies were negative.

Gastrointestinal tract

The glandular epithelium of the large bowel and the appendix showed a strong diffuse cytoplasmic, mainly subnuclear (large intestine) and supranuclear (appendix), immunostaining in the enterocytes. No clear differences were noticed among the different segments of the colon. The expression in the small intestine was focal cytoplasmic, mainly subnuclear in the duodenum and mainly peri-and supranuclear in the ileum. The antrum of the stomach showed a focal cytoplasmic, mainly subnuclear and brush border staining; the body showed a brush border and parietal cell expression. A strong positivity in foci of intestinal metaplasia in the gastric mucosa was the rule. The ductal epithelium of the eosophageal glands expressed the antigen as well. An expression by neuroendocrine cells throughout the gastrointestinal tract was generally obvious. The reactivity by basal cells in the epithelium of the esophagus and the anus was considered to refer to neuroendocrine cells. Strong positivity was found in the cells of the islets of Langerhans in the pancreas. The acinar cells of the exocrine pancreas was negative. Only some scattered positive cells were observed between them. The epithelium of the medium sized pancreatic ducts showed a cytoplasmic and mainly brush border immunostaining. Hepatocytes were negative. A cytoplasmic and brush border immunostaining was observed in the bile ducts and the gallbladder mucosa.

Respiratory tract

A mainly brush border staining by the respiratory epithelium of the bronchus, larynx, trachea, rhinopharynx and paranasal sinuses was observed. The staining in the ducts was cytoplasmic. The alveoli of the lung were negative.

Salivary glands (major and minor) and skin appendages

A cytoplasmic positivity in the ductal epithelium and scattered positive cells in the alveoli was noticed.

Spleen, tonsils, lymph nodes, bone marrow

Some positive cells, possibly dentritic, in the germinal centers of the follicles, mainly in the spleen were found. Various inflammatory cells, mainly the neutrophils but also the plasma cells, also showed positivity.

Thymus

A strong positivity in the Hassall's corpuscles was characteristic.

Adrenal gland

A weak to moderate positivity was observed in the medulla.

Thyroid gland

A focal protein immunoexpression was revealed by follicular cells, mainly in hyperplastic conditions and in oxyphilic cell metaplasia.

Parathyroids

An immunoexpression by the oxyphilic cells was noticed.

Pituitary gland

In the anterior pituitary many cells expressed the protein strongly. The pituitocytes of the pars nervosa were negative.

Mesothelium (pleura)

The positivity was variable and concerned mainly hyperplastic conditions.

Squamous epithelia

Squamous epithelia were generally negative. In some cases a weak focal expression by keratinocytes (cervix, mouth mucosa) was observed but could not be considered safely as positivity.

Mesenchymal tissues

Generally, mesenchymal tissues, except nervous tissue, were negative. Some weak positivity was observed in some myoepithelial cell in the wall of small arteries and by chondrocytes.

Diffuse neuroendocrine system

Neuroendocrine cells in several organs expressed hK6.

In short, hK6 is expressed by many normal human tissues. Glandular epithelia constitute the main hK6 immunoexpression sites, with representative organs being breast, prostate, kidney, endometrium, colon, appendix, salivary ducts, bile ducts and gallbladder. The small intestine, the stomach, the endocervix, the fallopian tube, the epididymis, the bronchus, and the upper respiratory tract show focal expression. Choroid plexus epithelium, peripheral nerves and neuroendocrine cells (including the islets of Langerhans and adrenal medulla) express the protein strongly and diffusely. A characteristic immunostaining is observed in the Hassall's corpuscles of the thymus, the oxyphilic cells in the thyroid and parathyroid glands, in the premordial follicles of the ovary, in dentritic cells mainly in the spleen and in various cells of the placenta.

DISCUSSION

The kallikrein 6 gene (KLK6) is one of the recently cloned members of the human tissue kallikrein gene family (Diamandis et al. 2000a). Messenger RNA encoding for hK6 protein (zyme/protease M/neurosin) has been previously detected in many tissues, including breast, brain, spinal cord, cerebellum, kidney, uterus, salivary gland, thymus, spleen, and testis in some mammalian species but not in mice, rats, or hamsters (Little et al. 1997; Anisowicz et al. 1996; Yamashiro et al. 1997; Yousef et al. 1999). Until recently, no methods were available for the quantitative measurement of hK6 in biological fluids and tissues. The development of polyclonal antibodies and of an immunofluorometric procedure for quantifying hK6 protein has helped to define the organs that express this protein and its relationship to cancer and other pathological conditions (Diamandis et al. 2000c; Diamandis et al. 2000d; Diamandis et al. 2000e). Recombinant hK6 protein was used for the development of polyclonal rabbit and mice antibodies and monoclonal mouse antibodies. The developed immunoassay for hK6 (Diamandis et al. 2000c) demonstrates good sensitivity and dynamic range and detects a single immunoreactive band in all biological fluids examined. This proteinase is present in its free form in serum, in contrast to PSA, which is mainly bound to α_1-antichymotrypsin (Diamandis et al. 2000c; Stenman et al. 1991).

This is the first report describing immunohistochemical localization of hK6 in a large variety of human tissues. Only Little et al. have previously studied hK6 immunohistochemically, and only in brain tissues (Little et al. 1997

hK6 was localized in a large number of normal human tissues and therefore, it cannot be considered as a specific tissue marker, in contrast to the homologous proteins hK2 and PSA which show prostate-restricted specificity (McCormack et al. 1995; Rittenhouse et al. 1998). With the availability of highly sensitive techniques, it has become apparent that these two kallikreins are also expressed in many other tissues including breast, thyroid and salivary glands (Black and Diamandis 2000; Magklara et al. 2000). The expression of hK6 mainly by glandular

epithelia suggests that it is secreted. This is further supported by the finding of hK6 in many biological fluids (Diamandis et al. 2000c). The absence of hK6 in urine corresponds well with the lack of immunoreactivity by the urothelium. The positive immunoexpression of glial cells in the study is in accordance with previous findings (Little et al. 1997; Yamanaka et al. 1999). Yamanaka et al. found KLK6 mRNA in mature oligodendrocytes and suggested that this protease may be important in the processes occurring after maturation of oligodendrocytes, such as myelination or turnover of the proteins in the myelin (Yamanaka et al. 1999). Little et al. detected positive immunostaining in monkey cortex cells lining the perimeter of cortical microvessels, in human brains of patients with Alzheimer's disease and in microglial cells, indicating a role of this protease in brain disease (Little et al. 1997). hK6 was also localized in the choroid plexus epithelium and correlated with Alzheimer's disease. Yamashiro et al. found strongest KLK6 mRNA expression in brain, followed by expression in spleen (Yamashiro et al. 1997), in accordance with the immunoreactivity of the Hassall's corspscules of the spleen in our study. An interesting finding is the expression of hK6 in oxyphilic cells in the thyroid and parathyroid glands. This accords with demonstration of hK2 and hK3 in oxyphilic cells of the thyroid (Magklara et al. 2000), suggesting another similarity between these three kallikreins. The detection of hK6 in human male and female serum (Diamandis et al. 2000c) is indicative that it is secreted by various tissues. Worth mentioning is the previous demonstration of other kallikreins, including hK3 (PSA) and hK2, in breast secretions, breast cancer cytosols, seminal plasma and amniotic fluids (Black and Diamandis 2000; Yu and Diamandis 1995a; Black et al. 2000; Yu and Diamandis 1995b).

Another interesting finding was the presence of hK6 in some cells of the diffuse neuroendocrine system and in nerves. The staining was relatively strong and it could be representative of neuroendocrine differentiation. The expression of hK6 is reminiscent of CD56 (natural killer cell associated antigen, neural cell adhesion molecule) and CD57 (Leu-7, T-cell surface marker) which are sensitive but not specific for cells and neoplasms with neuroendocrine differentiation (Kaufmann et al. 1997). Strong hK6 expression was found in the islets of Langerhans and in the epithelium of the pancreatic ducts, but not in the acinar cells of the exocrine pancreas. It is possible that hK6 may be involved in islet hormone processing, a property already attributed to many other proteolytic enzymes (Seidah and Chretien 1999).

The contribution of hK3 (PSA) and hK2 in the diagnosis and monitoring of prostate cancer suggests that other kallikreins may also have value as candidate biomarkers. Serum hK6 concentration has also been found to be increased in ovarian carcinoma (Diamandis et al. 2000d). Some other kallikrein genes are either underexpressed or overexpressed in certain carcinomas (Yousef and Diamandis 2001). The encoded proteases may serve to positively or negatively regulate cell growth or differentiation by cleavage of cell surface receptors (Coughlin 1999) or cell growth regulatory and angiogenic proteins, and by activation of other proteases for invasion and metastasis. These serine proteases are secreted into the extracellular space and therefore, they are excellent candidate circulating tumor markers. Beside KLK2 and KLK3, other kallikrein genes have been studied in relation to oncogenesis and prognosis of malignant tumors (Diamandis et al. 2000a; Yousef and Diamandis 2001). Liu et al. reported down-regulation of KLK10 in cancerous breast and prostate tissues and in cancer cell lines (Liu et al. 1996). Others reported up-regulation of TADG-14/KLK8 and KLK7 in ovarian carcinomas (Underwood et al. 1999; Tanimoto et al. 1999). Breast, prostate, renal and gastrointestinal cancer may reveal some relation with hK6 expression, as these organs strongly express this protein.

KLK6 has been shown to be regulated by steroid hormones (Yousef et al. 1999). Some kallikreins might be good therapeutic targets and the enzymatic activity of these proteins might be beneficial or deleterious. The development of tissue kallikrein inhibitors or activators may provide a new generation of drugs against cancer and other disorders.

Example 2

KLK6 Ovarian Tissue

PATIENTS AND METHODS

Ovarian Cancer Patients. One hundred eighty patients with primary ovarian cancer were included in this study. These patients underwent surgery for ovarian cancer at the Department of Gynecology, University of Turin, Italy. Patient age ranged from 25 to 82 years with a median of 59 years. Clinical and pathological information documented at the time of surgery included clinical stage of the cancer, grade and histology of the tumor, and amount of remaining tumor. Menopausal status was documented and response to chemotherapy monitored. Tumors were staged according to the International Federation of Gynaecology and Obstetrics (FIGO) criteria. Histologic classification was based on the World Health Organization and FIGO recommendations. Of the tumors included in this study, 80 were classified as serous papillary, 32 as undifferentiated, 27 as endometrioid, 13 as mucinous, 14 as clear cell, 10 as mullerian and 4 as other. The size of the residual tumors ranged from 0 to 9 cm, with a median of 1.1 cm.

Patients were monitored for survival and disease progression (no apparent progression or progression) for a median duration of 62 months (range 1-99 months). Follow-up information was available for 165 of the patients. 97 (54%) of these relapsed and 61 (34%) died during the course of the follow-up period.

Investigations were carried out in accordance with the ethical standards of the Helsinki Declaration of 1975, as revised in 1983, and were approved by the Institute of Obstetrics and Gynecology, Turin, Italy.

Preparation of Tumor Cell Extracts. Tumor tissue was frozen in liquid nitrogen immediately after surgery and stored at -80^0C until extraction. 20 to 100 mg of frozen tissue was pulverized on dry ice to a fine powder and added to 10 volumes of extraction buffer (50 mM Tris, pH 8.0, 150 mM NaCl, 5 mM EDTA, 10g/L of NP-40 surfactant, 1 mM phenylmethyl sulphonyl fluoride, 1g/L of aprotinin, 1g/L of leupeptin). The resulting suspension was incubated on ice for 30 minutes during which time it was vortexed every ten minutes. The mixture was then centrifuged at 14,000 rpm at 4^0C for 30 minutes and the supernatant (cell extract) was collected and stored at -80^0C until analysis. Protein concentration of the extract was determined with the bicinchoninic acid method, with albumin as standard (Pierce Chemical Co., Rockford, IL).

Measurement of hK6 in Ovarian Cell Extracts. The concentration of hK6 in tumor cell extract was quantified with a highly sensitive and specific non-competitive immunoassay for hK6 that has been previously described and evaluated in detail (Diamandis, E. P., Yousef, G. M., Soosaipillai, A. R., Grass, L., Porter, A., Little, S., and Sotiropoulou G. Immunofluorometric assay of human kallikrein 6 (Zyme/Protease M/Neurosin) and preliminary clinical applications. Clin. Biochem., 33: 369-375, 2000). The assay incorporated two hK6-specific polyclonal antibodies, one raised in mouse and the other in rabbit, in a sequential two site immunometric format with time resolved fluorescence detection. Analysis of standards, tumor cell extracts and control pools was carried out in duplicate in 96-well polystyrene microtiter plates with 200 µL of specimen added to the immunoassay. The standard curve using recombinant hK6 protein ranged from 0.5 µg/L to 200 µg/L. Assay precision was better than

10%. Signal detection and data reduction were performed automatically by the CyberFluor 615 Immunoanalyzer.

Localization of hK6 in Ovarian Tumor Specimens by Immunohistochemistry. A rabbit polyclonal antibody was raised against hK6 full-size recombinant protein, produced in yeast cells. Immunohistochemical staining for hK6 was performed according to a standard immunoperoxidase method. Briefly, paraffin-embedded tissue sections (4 μm) were fixed and dewaxed. Endogenous peroxidase activity was blocked with 3% aqueous hydrogen peroxide for 15 minutes. Sections were then treated with 0.4% pepsin at pH 2.0 for 5 minutes at 42°C and blocked with 20% protein blocker (Signet Labs) for 10 minutes. The primary antibody was then added at 1:400 dilution for 1 hour at room temperature. After washing, biotinylated anti-rabbit antibody (Signet) was added, diluted 4-fold in antibody dilution buffer (DAKO). Following incubation and washing, streptavidin tagged horseradish peroxidase was added for 30 minutes at room temperature. After washing, detection was achieved with amino ethyl carbazole (AEC) for 5-10 minutes. The slides were counterstained with hematoxylin and then mounted with cover slips.

Statistical Analysis. Statistical analysis was performed with SPSS software (SPSS Inc. Richmond, CA). To analyze data, patients were divided into different groups according to clinical and pathological parameters. Because the distribution of hK6 mass per mg total protein (i.e. specific activity) in the ovarian tumor extracts was not Gaussian, the non-parametric Mann-Whitney U test was used to determine differences between two groups and the non-parametric Kruskal-Wallis test was used for the analysis of differences among more than two groups. These tests treated hK6 specific activity in the tumor extract (ng hK6/mg total protein) as a continuous variable. hK6 tumor extract specific activity was also classified as either hK6-positive (> 35 ng/mg total protein; see Figure 1B for explanation) or hK6-negative (≤ 35 ng/mg total protein). The relationship of this dichotomous variable to other clinicopathological correlates was established with the Chi Square (χ^2) test or the Fisher's Exact Test, as appropriate. The impact of tumor extract hK6 specific activity on patient survival and on progression of the disease (progression-free survival) was assessed with the hazards ratio calculated by both univariate and multivariate Cox proportional hazards regression models (Cox, D. R. Regression tables and life tables. J. R. Stat. Soc. B, 34: 187-202, 1972). In the multivariate analysis, the clinical and pathological variables that may affect survival, including stage of disease, tumor grade, residual tumor, histologic type and age were adjusted. Kaplan-Meier progression-free survival and overall survival curves (Kaplan, E. L., and Meier, P. Nonparametric estimation from incomplete observations. J. Am. Stat. Assoc., 53: 457-481, 1958) were constructed to demonstrate the survival differences between the hK6-positive and hK6-negative patients. The log rank test (Mantel, N. Evaluation of survival data and two new rank order statistics arising in its consideration. Cancer Chemother. Rep., 50: 163-170, 1966) was used to examine the significance of the differences among the survival curves. Following analysis of the entire patient data set as a whole, the process was repeated on subgroups stratified separately by disease stage, by tumor grade and by amount of tumor remaining following surgery (debulking success). The impact of tumor hK6 level (positive or negative) on survival and on disease progression was determined by univariate and multivariate models for each of the subgroups.

RESULTS

Distribution of hK6 Specific Activity in Ovarian Tumor Extracts. The distribution of hK6 specific activity in ovarian tumor extracts from the 180 patients (Figure 1A) ranged from 0.04 ng/mg total protein to 497 ng/mg of total protein with a mean of 33 ng/mg total protein and a median of 13.2 ng/mg total protein. A value of 35

ng/mg total protein was identified by Chi square analysis (χ^2 = 7.3; P = 0.007) as the optimal cutpoint to distinguish positive from negative tumors in terms of predicting overall survival (Figure 1B). Thirty percent of the tumors were hK6 positive by this criterion. hK6 specific activity in tumor extracts was treated both as a continuous variable and as a dichotomous variable (≤ 35 ng/mg total protein, > 35 ng/mg total protein) in the analyses that follow.

hK6 specific activity (ng hK6/mg total protein) was significantly elevated (P < 0.001 by the Kruskal Wallis test) in extracts of ovarian tumor (mean 32.7, standard error 3.8, range 0.04 to 497) compared to extracts prepared from normal ovarian tissues (mean 3.5, standard error 2.5, range 0.05 to 20.8) or from ovarian tissue with benign disease (mean 3.2, standard error 2.6, range 0.03 to 21.5) (Figure 2). Further analysis showed there was no significant difference in hK6 specific activity among the ovarian tumors when they were stratified by histotype (i.e. serous vs undifferentiated vs endometrioid, etc).

Relationships between hK6 Status and Other Clinicopathological Variables. The distributions of various clinicopathological variables between hK6-positive and hK6-negative patients are summarized in Table 3. The relationships between hK6 status and these variables were examined with either the χ^2 Test or Fisher's Exact Test, as appropriate. No relationship was observed between hK6 status and tumor grade, menopausal status and response to chemotherapy. However, hK6-positive patients were more likely to have advanced disease (stage II-IV), serous tumor histology and greater residual tumor (>1 cm) (all P<0.05). hK6 tumor extract specific activity when treated as a continuous variable also associated proportionally with stage of the disease. Figure 3 shows the distribution of hK6 specific activity stratified by disease stage. hK6 specific activity was significantly higher in extracts from stage III/IV ovarian cancer than in those from stage I/II (P = 0.002 by the Mann Whitney U Test).

Univariate and Multivariate Survival Analysis. The impact of hK6 specific activity, other clinicopathological variables and age on disease progression and on overall survival is presented in Table 4. In univariate analysis, hK6-positive patients had a significantly increased risk of disease progression (hazard ratio =1.71) and death (hazard ratio =1.88) (P<0.05). When hK6 specific activity was treated as a continuous variable, hazard ratios were closely similar to those of hK6 negative tumors (arbitrarily set at 1.00), although the slight increase in risk of disease progression (hazard ratio = 1.005) was highly significant at P = 0.001. Kaplan-Meier survival curves demonstrated survival differences between hK6-positive and hK6-negative patients. As Figure 4 shows, the probability of progression-free and overall survival, respectively, are lower in hK6-positive patients than in hK6-negative patients.

The adverse effects of hK6 positivity on progression free survival and on overall survival were lost in multivariate analysis. As shown in Table 4, when survival outcomes were adjusted for other clinicopathological variables, hK6-positive and hK6-negative patients had statistically similar rates of disease progression and overall survival. Tumor grade also lost its univariate prognostic significance in multivariate analysis. Only stage of disease and residual tumor remaining after surgery maintained their independent effects on survival outcome in the multivariate analysis.

Univariate and Multivariate Survival Analysis in Subgroups of Patients. The patients were divided into different subgroups based on disease stage, tumor grade, and debulking success (residual tumor). In each subgroup, the impact of hK6 positivity and negativity on disease progression and on overall survival was determined by univariate and by multivariate Cox proportional hazard regression models. The results are shown

in Table 5. hK6 specific activity (positive, negative) significantly impacted survival in the subgroup of patients with tumor grade I or II. Univariate analysis revealed that hK6-positive patients were about 9-times more likely to suffer disease progression and 5-times more likely to die than hK6-negative patients. These survival differences remained significant even after the data were subjected to multivariate analysis. The relative risk of both outcomes arising from hK6 positivity was now about 4-fold ($P < 0.03$). hK6 status had no such effect among patients with Grade III tumor, nor could any discernible effect be demonstrated among patients with early stage disease and among those with greater than 1 cm of tumor remaining following surgery. Univariate analysis revealed a 2-fold increase in risk of disease progression and of death in the subgroup of patients with advanced disease (stage III and IV) who were hK6 positive, but the effect was lost in the multivariate analysis. The opposite occurred in the subset of patients characterized by optimal debulking of the tumor at the time of surgery (remaining tumor less than 1 cm in diameter). hK6 positivity had no demonstrable adverse effect on disease progression or on survival by univariate analysis, but did become statistically significant, giving a 3.5 and 5.5-fold increase in adverse risk, respectively, when the data were subjected to multivariate analysis. The emergence of effects in the multivariate model when none are generated by the univariate model happens when the adjusted variables have no impact at all on the outcome. In the case here, this means that stage of disease, tumor grade, tumor histology and patient age had no prognostic potential on disease progression and overall survival in this particular subset of patients. Kaplan-Meier survival curves of the subset of patients with grade I or II ovarian tumor are shown in Figure 5. As expected from the univariate analysis mentioned earlier, there was a significant difference in disease progression and survival between hK6 positive and hK6 negative patients.

Immunohistochemical Staining of hK6 in Ovarian Tumors. Immunohistochemical staining of hK6 in paraffin embedded tumor sections was roughly proportional to hK6 specific activity in tumor extracts (data not shown). The immunohistochemical localization of hK6 protein in four ovarian tissues that contained benign, borderline or malignant tumor is depicted in Figure 6. hK6 staining was restricted to epithelial cells, being absent in mesenchymal elements including fibrous supporting stroma. hK6 stained within the cytoplasm of epithelial cells, but staining intensity was variable among and within tumor preparations.

DISCUSSION

Increased hK6 synthesis was found to be predictive of more aggressive tumor behavior over time. Considered apart from other clinicopathological variables and age, hK6 positivity across the entire patient population under study was associated with about a 2-fold increase in the risk of both disease progression and of death. This effect was lost when outcomes were adjusted for the other clinicopathological variables and age in multivariate analysis of the entire patient population, but not when the multivariate analysis was restricted to those patients with lower grade tumor and with less residual tumor remaining after surgery (<1 cm in diameter). Among the former subgroup of patients, hK6 positivity predicted about a 4-fold increase in the risk of disease progression and of death ($P < 0.03$) while corresponding hazard ratios in the latter subgroup were 3.75 and 5.5, respectively ($P < 0.02$). The data show that hK6 positivity has independent predictive potential in these two subgroups and gives insight into tumor behavior over time that cannot be gleaned from the clinical parameters and pathological correlates conventionally measured. Hence hK6 testing could contribute to more individualized effective treatment of such patients.

hK6 is frequently overexpressed in ovarian tumors compared to nonmalignant ovarian tissue. This

overexpression tended to be higher in tumors from late stage disease than from early stage disease. The histochemical studies suggest that hK6 is synthesized by the epithelial cells of the ovary and is distributed diffusely within the cytoplasmic compartment.

Epithelial ovarian cancer has one of the worst prognoses among gynecologic malignancies, largely because over three-quarters of the diagnoses are made at a time when the disease has already established regional or distant metastases (Gatta, G., Lasota, M. B., and Verdecchia, A. Eur. J. Cancer, 34: 2218-2225, 19). Compounding the problem, tumor progression and aggressiveness correlate variably with conventional clinical and pathological markers. Thus there is an important need for additional diagnostic and prognostic markers for this disease and a number of potential markers have been identified. Molecular genetic analysis has uncovered several genes that are altered in a significant fraction of ovarian tumors (Aunoble, B., Sanches, R., Didier, E., and Bignon, Y. J., Int. J. Oncol., 16: 567-576, 2000. Shigemasa, K., Tanimoto, H., Parham, G. P., Parmley, T. H., Ohama, K., and O'Brien, T. J., J. Soc. Gynecol. Investig., 6: 102-108, 1999) and has identified other genes that appear to be involved in tumor progression (Suzuki, S., et al., Cancer Res., 60: 5382-5385, 2000). A whole host of serine proteases (Shigemasa, K., et al. J. Soc. Gynecol. Investig., 7: 358-362, 2000; Tanimoto, H., et al, Cancer Res., 57: 2884-2887, 1997; Hirahara, F., et al, Gynecol. Oncol., 68: 162-165, 1998; Underwood, L. J., et al.,. Cancer Res., 59: 4435-4439, 1999) in addition to those of the kallikrein family (Diamandis, E. P., et al Trends Endocrinol. Metab., 11; 54-60, 2000; Tanimoto, H., et al., Cancer, 86: 2074-2082, 1999) are overexpressed by epithelial ovarian tumor cells. These may have prognostic potential insofar as they assist in degrading the extracellular barriers such as interstitial connective tissue and basement membrane that must be breached in order for tumor to invade adjacent tissue and metastasize (Aznavoorian, S., et al,. Cancer, 71: 1368-1383, 1993; Duffy, M. J. Clin. Exp. Metasis, 10: 145-155, 1992.).

Table 1

Table 1: Tissues studied for the immunohistochemical expression of human kallikrein 6

Organ	Special tissue	Nr of cases (134)	Nr of paraffin blocks (199)
Brain and pituitary gland	Cortex, medulla, cerebellum, choroid plexus, meninges, pituitary	2 (autopsy)	34
Spinal cord		3	3
Heart		3	3
Lymph nodes		3	3
Spleen		3	3
Bone marrow		3	3
Thymus		3	3
Tonsils		3	3
Thyroid gland		3	3
Parathyroid gland		3	3
Pituitary gland		3	3
Adenal gland	Cortex, medulla	3	3
Skin		3	3
Mouth mucosa		3	3
Paranasal sinuses		3	3
Rhinopharynx		3	3
Esophagus		3	3
Stomach	Fundus, body, antrum	3	9

Table 1 cont'd

Small intestine	Duodenum, jejunum, ileum	3	9
Large intestine	Cecum, ascending colon, transverse colon, descending colon, sigmoid, rectum	3	18
Anus		3	3
Appendix		3	3
Larynx		3	3
Broncus		3	3
Lung		3	3
Major salivary glands		3	3
Liver		3	3
Gallbladder		3	3
Pancreas		3	3
Kidney	Cortex, medulla	3	6
Urinary bladder		3	3
Prostate gland		3	3
Seminal vesicles		3	3
Cavernous bodies		3	3
Testis		3	3
Rete testis		3	3

Table 1 cont'd

Ejaculatory ducts	3		
Table 1 cont'd			
Epididymis	3	3	
Ovary	3	3	
Uterus	6	3	
Vagina	3	3	
Vulva	3	3	
Fallopian tube	3	3	
Placenta	3	3	
Breast	3	3	
Corpus, cervix			

Table 2

Table 2: Tissues with a hK6 immunoexpression

Tissue	Immunostaining characteristics
Choroid plexus	D*
Glial cells	D, weak
Peripheral nerves	D
Breast	C*, BB*, LS*
Endometrium	C (droplets), BB, LS
Endocervix	PN*, BB
Fallopian tube	SpN*, BB
Ovary	Premordial follicles
Placenta	Endothelia, X cells
Prostate	D
Epididymis, seminal vesicles, spermatic cord	SpN, BB
Kidney (urinary tubuli)	C
Colon, appendix	SbN* (colon), SpN* (appendix)
Small intestine	Focal C
Stomach	C, SbN, BB, parietal cells
Bile ducts, and gallbladder	C, BB
Pancreas	Islets of Langerhans
Bronchus, larynx, trachea, rhinopharynx, paranasal sinuses	BB, C (ducts)
Salivary glands	C

Spleen	Dentritic cells

Table 2 cont'd

Thymus	Hassall's corpuscles
Adrenal gland (medulla)	C
Thyroid gland	Focal, mainly in hyperplasia
Parathyroids	Oxyphilic cells
Diffuse neuroendocrine system	D

*D=diffuse, C=cytoplasmic, BB=brushing border, LS=luminal secretions, PN=paranuclear, SpN=supranuclear, SbN=subnuclear

Table 3. Relationship between hK6 status and other variables in 180 ovarian cancer patients.

Variable	Patients	No. of patients (%) hK6 negative	hK6 positive	P value
Stage				
I	44	38 (86.4)	6 (13.6)	
II	13	8 (61.5)	5 (38.5)	0.034[a]
III	110	72 (65.4)	38 (34.5)	
IV	13	7 (53.8)	6 (46.2)	
Grade				
G1	25	21 (84.0)	4 (16.0)	
G2	27	21 (77.8)	6 (22.2)	0.33[a]
G3	119	84 (70.6)	35 (29.4)	
X	9			
Histotype				
Serous	80	52 (65.0)	28 (35.0)	
Undifferentiated	27	17 (63.0)	10 (37.0)	
Endometrioid	32	27 (46.7)	5 (53.3)	
Mucinous	13	10 (76.9)	3 (13.1)	0.31[b]
Clear cell	14	11 (78.6)	3 (21.4)	
Mullerian	10	8 (80.0)	2 (20.0)	
Others	4	3 (75.0)	1 (25.0)	
Residual tumor (cm)				
0	80	67 (83.2)	13 (16.3)	
1-2	29	16 (55.2)	13 (44.8)	0.002[a]
>2	64	40 (62.5)	24 (37.5)	
x	7			
Menopause				
Pre/peri	50	32 (64.0)	18 (36.0)	0.075[b]
Post	130	99 (76.2)	31 (23.8)	
Response to CTX[c]				
NC/PD	15	11 (73.3)	4 (26.7)	0.99[b]
CR/PR	148	104 (70.3)	44 (29.7)	
NE	17			

[a] χ^2 test.
[b] Fisher's Exact Test
[c] CTX; chemotherapy, NC; no change, PD; progressive disease, CR; complete response, PR; partial response, NE; not evaluated.
x. Status unknown.

Table 4. Univariate and Multivariate Analysis of Prognostic Value of hK6

Variable	Progression-free survival (PFS)			Overall survival (OS)		
	HR[a]	95% CI[b]	P value	HR[a]	95% CI[b]	P value
	Univariate Analysis					
hK6						
Negative	1.00			1.00		
Positive	1.71	1.11-2.64	0.015	1.88	1.09-3.21	0.022
as a continuous variable	1.005	1.002-1.007	0.001	1.004	0.999-1.008	0.074
Stage of disease (ordinal)	2.79	2.07-3.79	<0.001	3.07	2.05-4.61	<0.001
Grading (ordinal)	1.95	1.38-2.75	<0.001	2.07	1.31-3.29	0.002
Residual tumor (ordinal)	1.27	1.20-1.34	<0.001	1.31	1.22-1.41	<0.001
Histologic type[c]	0.83	0.68-1.00	0.055	0.88	0.69-1.13	0.34
Age	1.012	0.99-1.03	0.14	1.015	0.99-1.03	0.15
	Multivariate Analysis					
hK6						
Negative	1.00			1.00		
Positive	1.40	0.84-2.32	0.19	1.08	0.79-1.49	0.62
as a continuous variable	1.002	0.99-1.006	0.22	1.001	0.99-1.004	0.69
Stage of disease (ordinal)	1.57	1.09-2.27	0.014	1.72	1.053-2.82	0.03
Grading (ordinal)	1.31	0.84-2.32	0.18	1.31	0.75-2.25	0.33
Residual tumor (ordinal)	1.14	1.05-1.24	0.001	1.21	1.09-1.34	<0.001
Histologic type[c]	0.95	0.82-1.11	0.57	1.04	0.86-1.26	0.68
Age	1.02	0.99-1.039	0.12	1.02	0.99-1.04	0.21

[a] Hazard ratio (HR) estimated from Cox proportional hazard regression model
[b] Confidence interval of the estimated HR.
[c] Serous vs. others

Table 5. Cox proportional hazard regression analysis for subgroups of patients

Variable	Progression-free survival			Overall survival		
	HR[a]	95% CI[b]	P value	HR[a]	95% CI[b]	P value
Tumor grade I-II						
hK6 univariate	9.25	3.33-25.67	<0.001	5.05	1.63-15.71	0.005
hK6 multivariate[c]	4.29	1.17-15.65	0.027	4.05	1.23-16.6	0.023
Tumor grade III						
hK6 univariate	1.45	0.87-2.39	0.14	1.69	0.91-3.14	0.091
hK6 multivariate[c]	1.03	0.58-1.83	0.91	1.02	0.48-2.13	0.96
Stage I-II						
hK6 univariate	0.90	0.18-4.35	0.89	1.49	0.13-16.53	0.74
hK6 multivariate[d]	1.83	0.17-19.41	0.61	2.23	0.20-25.04	0.51
Stage III-IV						
hK6 univariate	2.04	1.26-3.29	0.004	1.98	1.12-3.47	0.017
hK6 multivariate[d]	1.57	0.93-2.68	0.092	1.33	0.71-2.53	0.37
Optimal debulking success[e]						
hK6 univariate	1.81	0.72-4.55	0.20	2.61	0.70-9.73	0.15
hK6 multivariate[f]	3.75	1.39-10.09	0.019	5.57	1.47-21.04	0.011
Suboptimal debulking success[e]						
HK6 univariate	1.39	0.83-2.32	0.20	1.16	0.64-2.09	0.62
HK6 multivariate[f]	1.27	0.72-2.23	0.40	1.19	0.62-2.27	0.59

[a] Hazard ratio (HR) estimated from Cox proportional hazard regression model

[b] Confidence interval of the estimated HR.

[c] Multivariate models were adjusted for stage of disease, residual tumor, histologic type and age.

[d] Multivariate models were adjusted for tumor grade, residual tumor, histologic type and age.

[e] Optimal debulking (0 - 1 cm residual tumor); suboptimal debulking (> 1 cm residual tumor)

[f] Multivariate models were adjusted for stage of disease, tumor grade, histologic type and age.

Table 6

Kallikrein Protein	GenBank Accession No.
hK4	AF113141, AF135023, AF148532
hK5	AF135028, AF168768
hK6	AF013988, AF149289, U62801
hK7	L33404, AF166330
hK8	AB009849, AF095743, AB010780, AF055982
hK9	AF135026
hK10	AF055481, NM_002776
hK11	AB012917, AF164623
hK12	AF135025
hK13	AF135024
hK14	AF161221
hK15	AF242195

5

While the present invention has been described with reference to what are presently considered to be the preferred examples, it is to be understood that the invention is not limited to the disclosed examples. To the contrary, the invention is intended to cover various modifications and equivalent arrangements included within the spirit and scope of the appended claims.

All publications, patents and patent applications are herein incorporated by reference in their entirety to the same extent as if each individual publication, patent or patent application was specifically and individually indicated to be incorporated by reference in its entirety.

Below full citations are set out for the references referred to in the specification.

FULL CITATIONS FOR REFERENCES REFERRED TO IN THE SPECIFICATION

Anisowicz A, Sotiropoulou G, Stenman G. Mok SC, Sager R (1996) A novel protease homolog differentially expressed in breast and ovarian cancer. Mol Med 2:624-636

Black MH, Diamandis EP (2000) The diagnostic and prognostic utility of prostate specific antigen for diseases of the breast. Breast Cancer Res Treat 59:1-4

Black MH, Magklara A, Obiezu C, Levesque MA, Sutherland DJ, Tindall DJ, Young CY, Sauter ER, Diamandis EP (2000) Expression of a prostate-associated protein, human glandular kallikrein (hK2), in breast tumors and in normal breast secretions. Br J Cancer 82:361-367

Chu TM (1997) Prostate-specific antigen and early detection of prostate cancer. Tumor Biol 18:123-134

Coughlin SR (1999) How the protease thrombin talks to cells. Proc Natl Acad Sci USA 96:11023-11027

Diamandis EP (1999) Prostate specific antigen – its usefulness in clinical medicine. Trends Endocrinol Metab 25:14-16

Diamandis EP, Yousef GM, Luo LY, Magklara A, Obiezu CV (2000a) The new human kallikrein gene family: implications in carcinogenesis. Trends Endocrinol Metab 11:54-60

Diamandis EP, Yousef GM, Clements J, Ashworth LK, Yoshida S, Egelrud T, Nelson PS, Shiosaka S, Little S, Lilja H, Stenman U-H, Rittenhouse HG, Wain H (2000b) New nomenclature for the human tissue kallikrein gene family. Clin Chem 46:1855-1858

Diamandis EP, Yousef GM, Soosaipillai AR, Grass L, Porter A, Little S, Sotiropoulou G (2000c) Immunofluorometric assay of human kallikrein 6 (Zyme/Protease M/Neurosin) and preliminary clinical applications. Clin Biochem 33:369-375

Diamandis EP, Yousef GM, Soosaipillai AR, Bunting P (2000d) Human kallikrein 6 (zyme/protease M/neurosin): a new serum biomarker of ovarian carcinoma. Clin Biochem 33:579-583

Diamandis EP, Yousef GM, Petraki C, Soosaipillai AR (2000e) Human kallikrein 6 as a biomarker of Alzheimer's disease. Clin Biochem 33:663-667.

Kaufmann O, Georgi T, Dietel M (1997) Utility of 123C3 monoclonal antibody against CD56 (NCAM) for the diagnosis of small cell carcinomas on paraffin sections. Hum Pathol 28:1373-1378

Little SP, Dixon EP, Norris F, Buckley W, Becker GW, Johnson M, Dobbins JR, et al (1997) Zyme, a novel and potentially amyloidogenic enzyme cDNA isolated from Alzheimer;s disease brain. J Biol Chem 272:25135-25142

Liu XL, Wazer DE, Watanabe K, Band V (1996) Identification of a novel serine protease-like gene, the expression of which is down-regulated during breast cancer progression. Cancer Res 56:3371-3379

Maglara A, Cheung CC, Asa SL, Diamandis EP (2000) Expression of prostate-specific antigen and human glandular kallikrein 2 in the thyroid gland. Clin Chim Acta 300:171-180

McCormack RT, Rittenhouse HG, Finlay JA, Sokoloff RL, Wang TJ, Wolfert RL, Lilja H, Oesterling JE (1995) Molecular forms of prostate-specific antigen and the human kallikrein gene family: a new era. Urology 45:729-744

Rittenhouse HG, Finlay JA, Mikolajczyk SD, Partin AW (1998) Human kallikrein 2 (hK2) and prostate-specific antigen (PSA): Two closely related, but distinct, kallikreins in the prostate. Crit Rev Clin Lab Sci 35:275-368

Seidah NG, Chretien M (1999) Properties and phohormone convertases: a family of subtilases generating diverse bioactive polypeptides. Brain Res 848:45-62

Stenman U-H (1999) New ultrasensitive assays facilitate studies on the role of human glandular kallikrein (hK2) as a marker for prostatic disease. Clin Chem 45:753-754

Stenman U-H, Leinonen J, Alfthan H, Rannikko S, Tuhkanen K, Alfthan O (1991) A complex between prostate-specific antigen and alph1-antichymotrypsin is the major form of prostate-specific antigen in serum of patients with prostate cancer: assay of the complex improves clinical sensitivity for cancer. Cancer Res 51:222-226

Tanimoto H, Underwood LJ, Shigemasa K, Yan Yan MS, Clarke J, Parmley TH, O'Brien TJ (1999) The stratum corneum chymotryptic enzyme that mediates shedding and desquamation of skin cells is highly overexpressed in ovarian tumor cells. Cancer 86:2074-2782

Underwood LJ, Tanimoto H, Wang Y, Shigemasa K, Parmley K, O'Brien TJ (1999) Cloning of tumor-associated differentially expressed gene-14, a novel serine protease overexpressed by ovarian carcinoma. Cancer Res 59:4435-4439

Yamanaka H, He X, Matsumoto K, Shiosaka S, Yoshida S (1999) Protease M/neurosin mRNA is expressed in mature oligodendrocytes. Brain Res Mol Brain Res 71:217-224

Yamashiro K, Tsuruoiko N, Kodama S, Tsujimoto M, Yamamura T, Tanaka T, Nakazato H, Yamaguchi N (1997) Molecular cloning of a novel trypsin-like serine protease (neurosin) preferentially expressed in brain. Biochim Biophys Acta 1350:11-14

Yousef GM, Diamandis EP (2001) The new human tissue kallikrein gene family: structure, function and association to disease. Endocr Rev 22:184-204.

Yousef GM, Luo L-Y, Scherer SW, Sotiropoulou G, Diamandis EP (1999) Molecular characterization of zyme/proteaseM/neurosin (PRSS9), a hormonally regulated kallikrein-like serine protease. Genomics 62:251-259

Yu H, Diamandis EP (1995a) Prostate specific antigen in milk of lactating women. Clin Chem 41:54-58.

Yu H, Diamandis EP (1995b) Prostate specific antigen immunoreactivity in amniotic fluid. Clin Chem 41: 204-205

I Claim:

1. A method of evaluating an excised mammalian tissue sample for the presence of a tumor bearing a kallikrein, preferably an ovarian tumor, comprising the steps of: exposing the tissue to an amount of a detectably labeled kallikrein binding molecule or agent effective to bind to the kallikrein tumor tissue, and examining the sample for the presence or absence of labeled kallikrein.
2. An *in vivo* method comprising administering to a mammal one or more agent that carries a label for imaging and binds to a kallikrein, preferably hK6, and then imaging the mammal.
3. A method for imaging a tumor, preferably an ovarian tumor in a subject, the tumor having one or more of kallikrein 4, kallikrein 5, kallikrein 6, kallikrein 7, kallikrein 8, kallikrein 9, kallikrein 10, kallikrein 11, kallikrein 12, kallikrein 13, kallikrein 14, and kallikrein 15, comprising the steps of: delivering into the subject an amount of a detectably-labeled kallikrein binding molecule effective to image the tumor; and scanning the subject to determine the distribution of the labeled kallikrein.
4. A method as claimed in claim 3 wherein the tumor is an ovarian tumor having kallikrein 5 and kallikrein 6; kallikrein 4, kallikrein 5, kallikrein 6, kallikrein 7, and kallikrein 8; or, kallikrein 4, kallikrein 5, kallikrein 6, kallikrein 7, kallikrein 8, kallikrein 9, kallikrein 10, and kallikrein 11.
5. An *in vivo* method for imaging ovarian cancer is provided comprising:
   (a) injecting a patient with an agent that binds to kallikrein 6 the agent carrying a label for imaging the ovarian cancer;
   (b) allowing the agent to incubate *in vivo* and bind to kallikrein 6 associated with the ovarian cancer; and
   (c) detecting the presence of the label localized to the ovarian cancer.
6. A method as claimed in claim 5 further comprising in step (a) above, injecting the patient with one or more of an agent that binds to human stratum corneum chymotryptic enzyme (HSCCE, also known as kallikrein 7 or hK7), kallikrein 4, kallikrein 5, kallikrein 8, kallikrein 9, kallikrein 10, kallikrein 11, CA125, CA15-3, CA19-9, CA72-4, OVX1, lysophosphatidic acid (LPA) or carcinoembryonic antigen (CEA).
7. A composition adapted for imaging ovarian cancer in a patient or tissue sample comprising agents that bind to kallikrein 5 and kallikrein 6, or kallikrein 4, kallikrein 5, kallikrein 6, kallikrein 7, and kallikrein 8 wherein the agents carry a label for imaging, and the kallikrein is located in the patient or sample by visualizing the imaging agent bound to the kallikrein.
8. A pharmaceutical formulation comprising a composition comprising an agent that binds a kallikrein and a label for imaging a tumor, preferably an ovarian tumor, and a pharmaceutically acceptable excipient, and wherein the composition is present in an amount sufficient to enhance a computer assisted tomography (CAT) image, a magnetic resonance spectroscopy (MRS) image, a magnetic resonance imaging (MRI) image, a positron emission tomography (PET) image, a single-photon emision computed tomography (SPECT) image, or a bioluminescence image (BLI), or equivalents

thereof, when the pharmaceutical formulation is administered to an individual, or applied to a tissue or organ *in situ*, in a sufficient amount.

9. A method for *in situ* or *in vivo* imaging of a cell, tissue, an organ, or a full body comprising administering a pharmaceutical formulation as claimed in claim 8, in an amount sufficient to enhance an image, wherein the image is generated by computer assisted tomography (CAT), magnetic resonance spectroscopy (MRS) image, magnetic resonance imaging (MRI), positron emission tomography (PET), single-photon emision computed tomography (SPECT), or bioluminescence imaging (BLI), or equivalents thereof.

10. A method of *in situ* or *in vivo* imaging of a cell, a tissue, an organ or a full body comprising the following steps: (a) providing a pharmaceutical formulation as claimed in claim 8; (b) providing an imaging device, wherein the imaging device is computer assisted tomography (PET), magnetic resonance spectroscopy (MRS), magnetic resonance imaging (MRI), a positron emission tomography (PET), single-photon emision computed tomography (SPECT), or bioluminescence imaging (BLI) or equivalent; (c) administering the pharmaceutical formulation in an amount sufficient to generate the cell, tissue, or body image; and (d) imaging the distribution of the pharmaceutical formulation of step (a) with the imaging device, thereby imaging the cell, tissue or body.

11. A method as claimed in claim 10 wherein the pharmaceutical formulation is administered to a human, such as a cancer patient, in particular an ovarian cancer patient, or a patient suspected of having or being screened for cancer, in particular ovarian cancer.

12. A method as claimed in claim 10 wherein the pharmaceutical formulation is administered intravenously.

13. A method for *in vivo* imaging tumor neovasculature, preferably ovarian tumor neovasculature, in a subject comprising: (a) providing a pharmaceutical formulation as claimed in claim 8; (b) providing an imaging device, wherein the imaging device is computer assisted tomography (CAT), magnetic resonance spectroscopy (MRS), magnetic resonance imaging (MRI), positron emission tomography (PET), single-photon emission computed tomography (SPECT), bioluminescence imaging (BLI) or equivalent; (c) administering the pharmaceutical formulation in an amount sufficient to image the tumor neovasculature; and, (d) imaging the distribution of the pharmaceutical formulation of step (a) with the imaging device, thereby imaging the tumor neovasculature.

14. A method for *in situ* or *in vivo* screening for an anti-tumor agent by imaging tumor neovasculature, preferably ovarian tumor neovasculature, in an individual comprising the following steps: (a) providing a composition comprising a pharmaceutical formulation as claimed in claim 8, and a test compound; (b) providing an imaging device, wherein the imaging device is computer assisted tomography (CAT), magnetic resonance spectroscopy (MRS), magnetic resonance imaging (MRI), positron emission tomography (PET), single-photon emission computed tomography (SPECT), bioluminescence imaging (BLI) or equivalent; (c) administering the composition of step (a) in an amount sufficient to image the tumor and imaging the distribution of the composition with the imaging device, thereby imaging the tumor; (d) administering the test compound; and, (e) imaging the distribution of the composition with the imaging device, thereby imaging the tumor, wherein a

decrease in the amount of tumor neovasculature indicates that the compound is an anti-tumor or an anti-angiogenic agent.

15. A method as claimed in any preceding claim wherein the agent or kallikrein binding molecule is an antibody that recognizes or binds to a kallikrein.

16. A method as claimed in any preceding claim wherein the label is a radiolabel, fluorescent label, nuclear magnetic resonance active label, positron emitting isotope detectable by a positron emission tomography ("PET") scanner, chemiluminescer, bioluminescent label, or enzymatic marker.

17. A kit for carrying out a method as claimed in any preceding claim.

Figure 1A

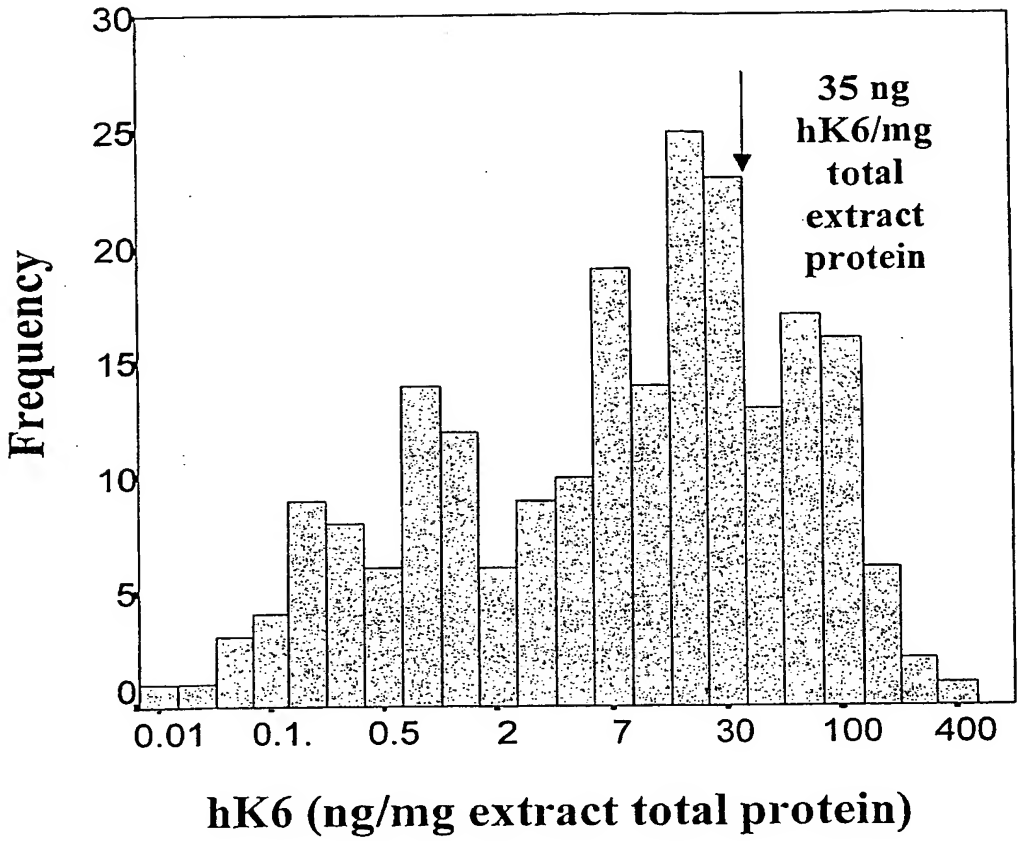


Figure 1B

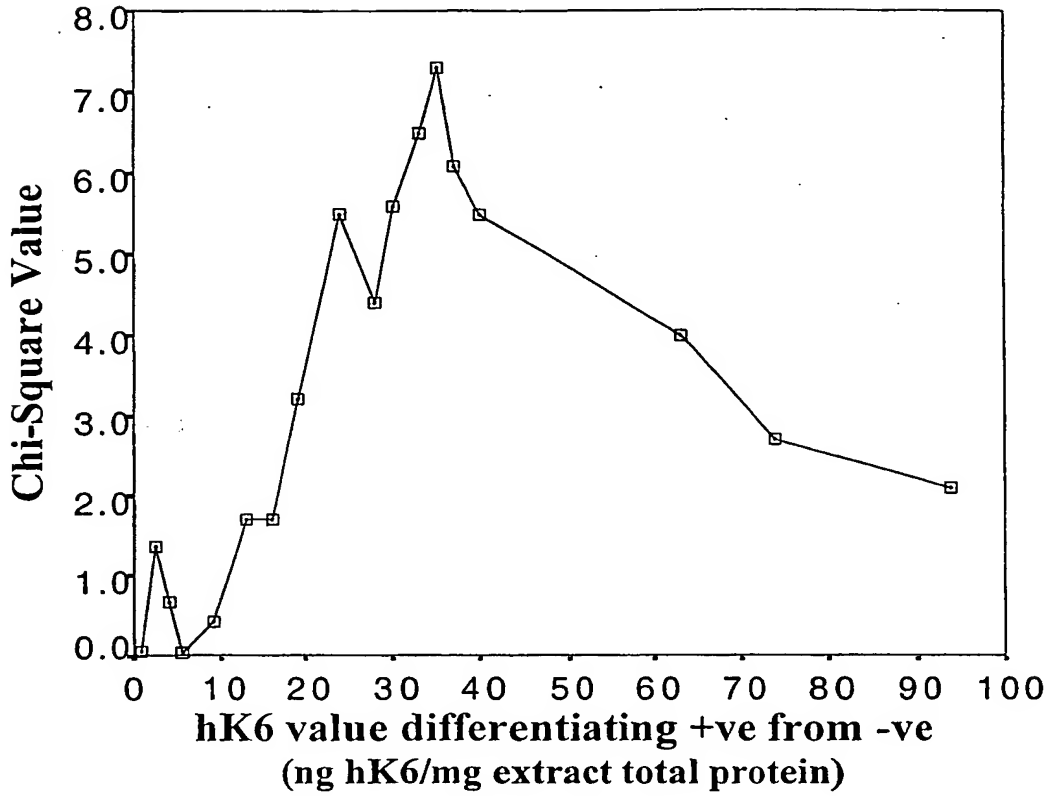

Chi-Square Value
8.0
7.0
6.0
5.0
4.0
3.0
2.0
1.0
0.0
0
10
20
30
40
50
60
70
80
90
100
hK6 value differentiating +ve from -ve
(ng hK6/mg extract total protein)

Figure 2

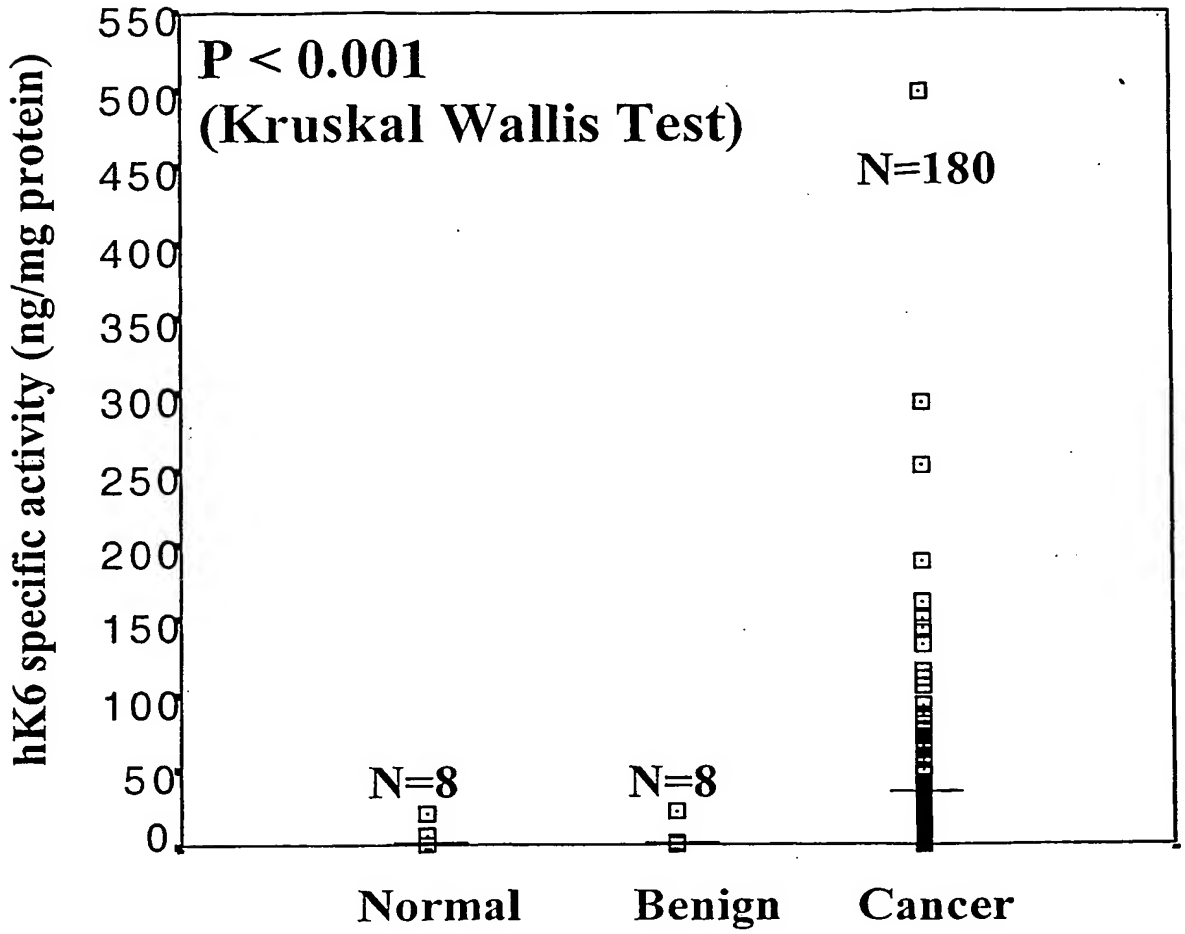


Figure 3

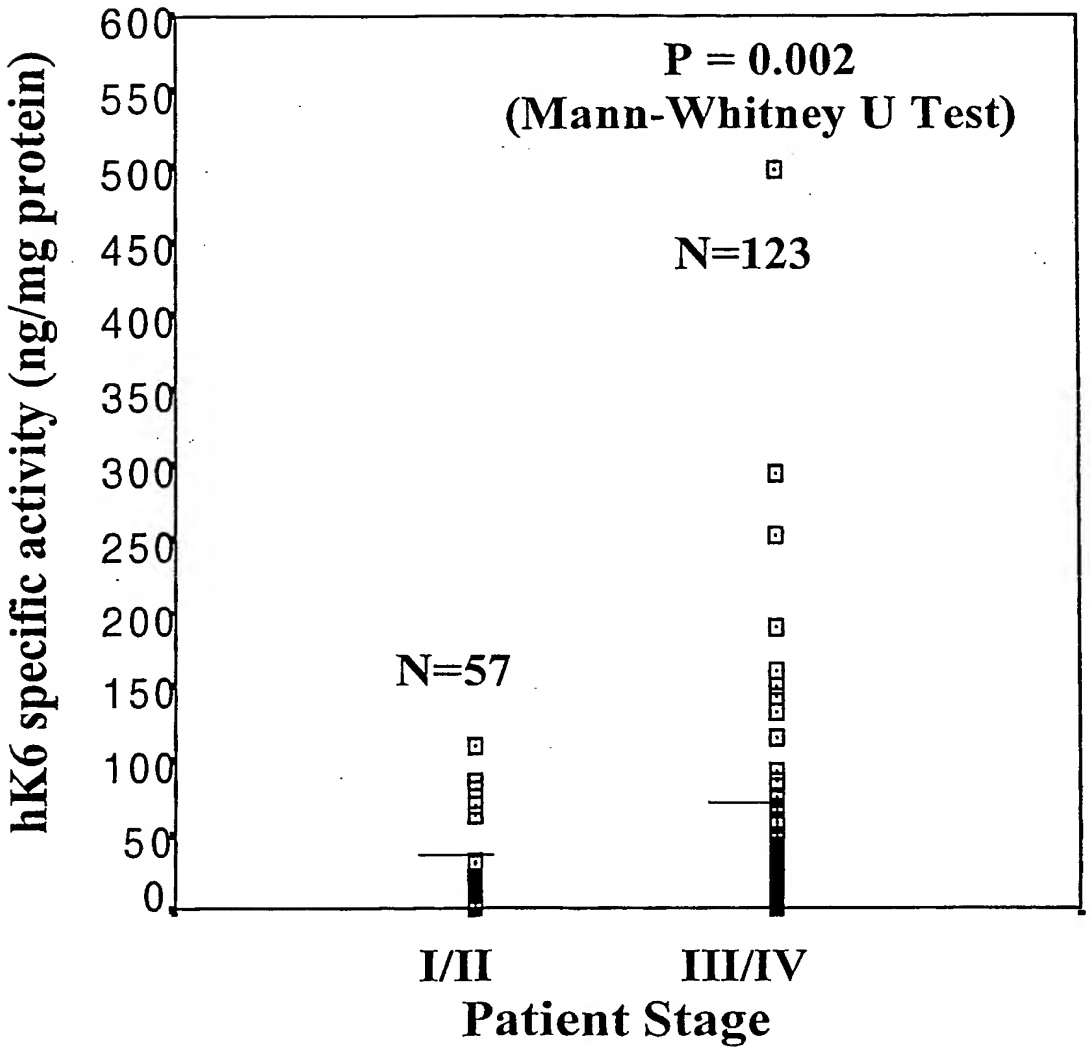
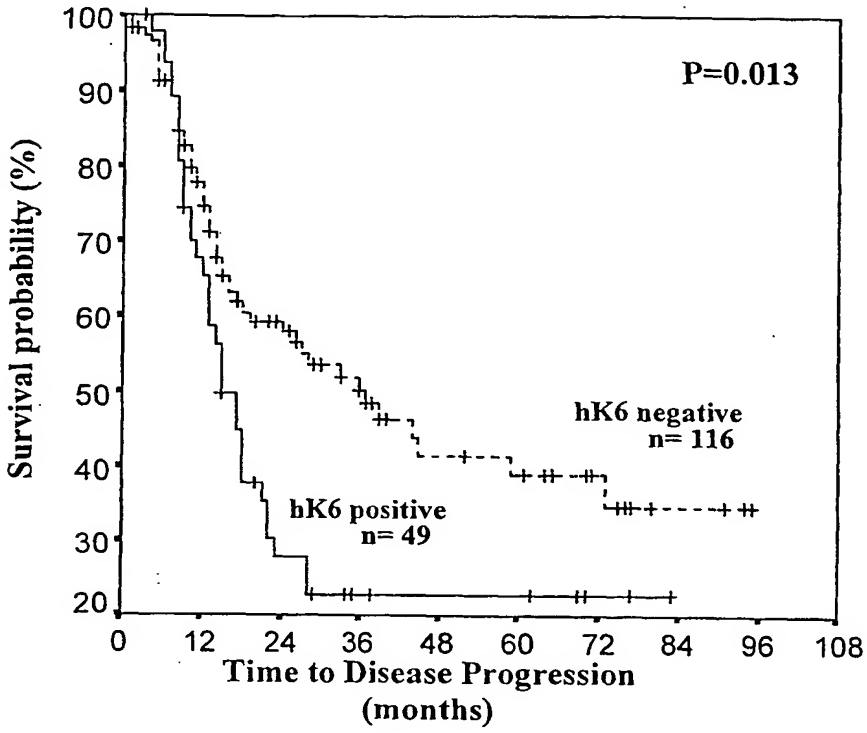
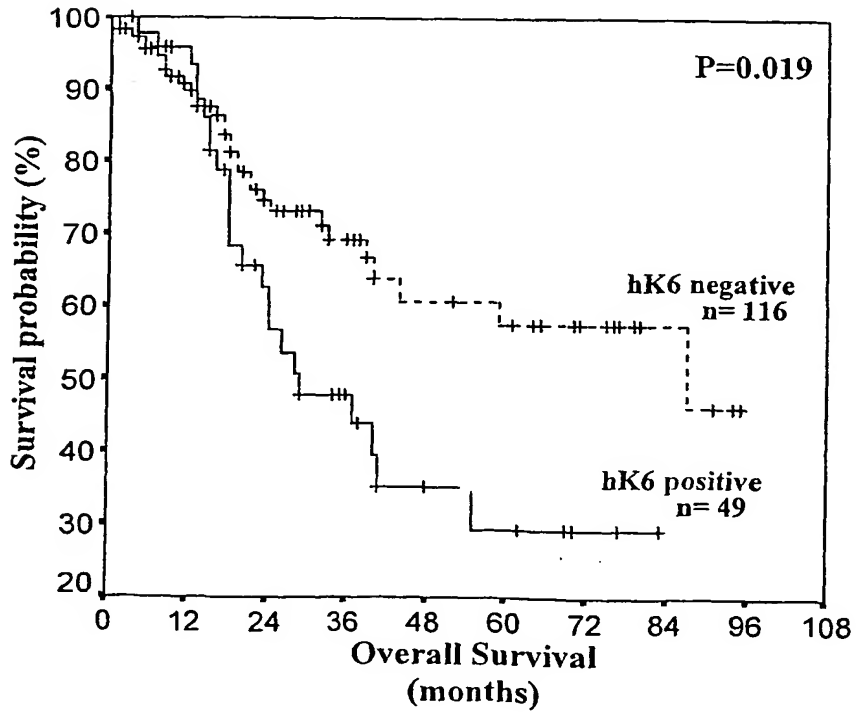

hK6 specific activity (ng/mg protein)
600
550
500
450
400
350
300
250
200
150
100
50
0
P = 0.002
(Mann-Whitney U Test)
N=123
N=57
I/II
III/IV
Patient Stage

Figure 4





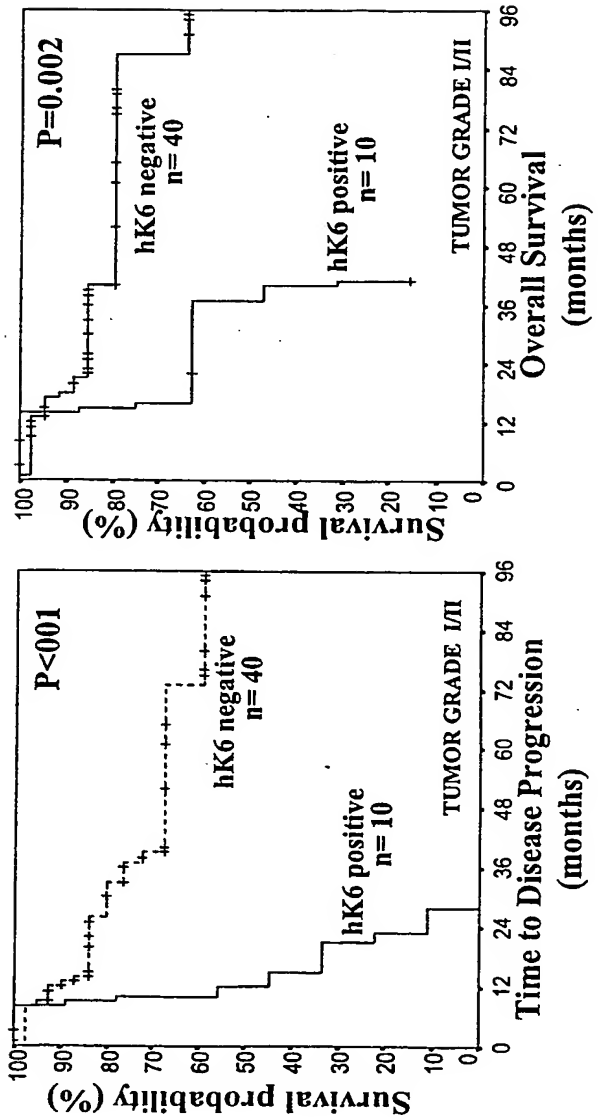
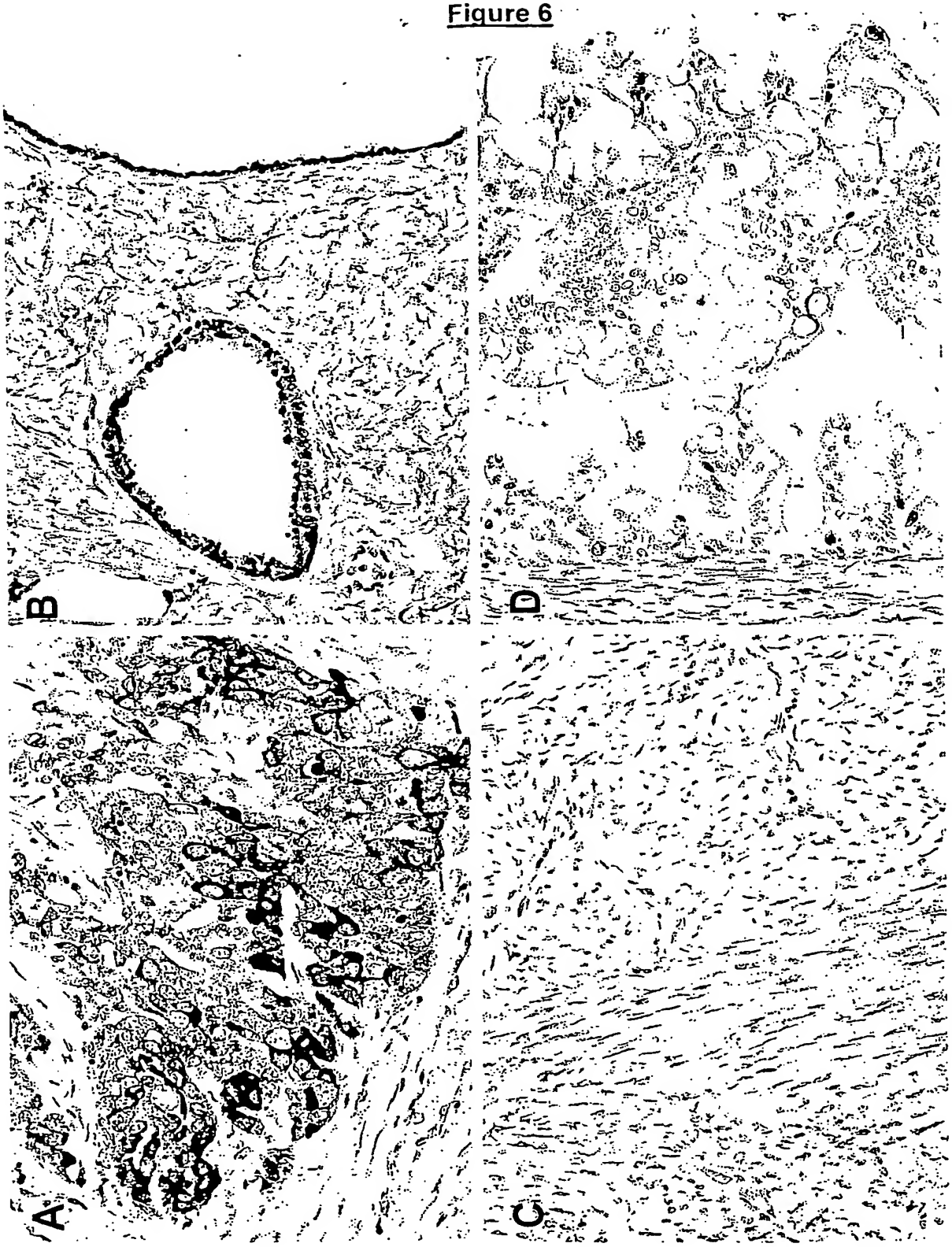

P<001
hK6 negative
n= 40
hK6 positive
n= 10
TUMOR GRADE I/II
Survival probability (%)
Time to Disease Progression
(months)
P=0.002
hK6 negative
n= 40
hK6 positive
n= 10
TUMOR GRADE I/II
Survival probability (%)
Overall Survival
(months)

Figure 5

Figure 6



INTERNATIONAL SEARCH REPORT

Inte.....al Application No
PCT/CA 02/01556

A. CLASSIFICATION OF SUBJECT MATTER
IPC 7 G01N33/574 A61K49/16

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 7 G01N A61K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)
EPO-Internal, WPI Data, BIOSIS, PAJ, EMBASE

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	WO 01 59158 A (TRUSTEES OF THE UNIVERSITY OF) 16 August 2001 (2001-08-16) page 33, line 3-10; figure 13 page 18, paragraph 2 page 58, line 25 -page 59, line 1 page 62, line 6-15; table 8	1-13, 15-17
X	WO 98 11238 A (DANA FARBER CANCER INST INC) 19 March 1998 (1998-03-19) page 3, line 25 - line 34 page 5, line 14 - line 25 page 5, line 32 -page 6, line 15 page 33, line 7 -page 36, line 9 page 53, line 4 -page 57, line 20	1,7,15, 17
Y	claims 33-46; figure 5; table 3	2-6, 8-14,16

-/--

[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

° Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
11 December 2002	09/01/2003

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax: (+31-70) 340-3016	Vanmontfort, D

Form PCT/ISA/210 (second sheet) (July 1992)

INTERNATIONAL SEARCH REPORT

Int I Application No
PCT/CA 02/01556

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication,where appropriate, of the relevant passages	Relevant to claim No.
X	TANIMOTO HIROTOSHI ET AL: "Increased expression of protease M in ovarian tumors." TUMOR BIOLOGY, vol. 22, no. 1, January 2001 (2001-01), pages 11-18, XP009002602 ISSN: 1010-4283	1,15
Y	the whole document	2-14,16
X	DIAMANDIS ELEFTHERIOS P ET AL: "Human kallikrein 6 (zyme/protease M/neurosin): A new serum biomarker of ovarian carcinoma." CLINICAL BIOCHEMISTRY, vol. 33, no. 7, October 2000 (2000-10), pages 579-583, XP002224577 ISSN: 0009-9120 cited in the application	1,15
Y	the whole document	2-14,16
X	LUO L Y ET AL: "Prognostic value of human kallikrein 10 expression in epithelial ovarian carcinoma" CLINICAL CANCER RESEARCH, THE AMERICAN ASSOCIATION FOR CANCER RESEARCH, US, vol. 7, no. 8, August 2001 (2001-08), pages 2372-2379, XP002211680 ISSN: 1078-0432	1,15
Y	the whole document	2,3, 8-14,16
Y	US 6 068 830 A (DIAMANDIS ELEFTHERIOS P ET AL) 30 May 2000 (2000-05-30) column 3, line 44 -column 4, line 25; claims 1-7; example 2	2-14,16
P,X	WO 02 35232 A (DIAMANDIS ELEFTHERIOS P ;MOUNT SINAI HOSPITAL (CA)) 2 May 2002 (2002-05-02) the whole document	1-17
P,X	DIAMANDIS ELEFTHERIOS P ET AL: "Human tissue kallikreins: A family of new cancer biomarkers." CLINICAL CHEMISTRY, vol. 48, no. 8, August 2002 (2002-08), pages 1198-1205, XP001121702 August, 2002 ISSN: 0009-9147 the whole document	1-17

Form PCT/ISA/210 (continuation of second sheet) (July 1992)

INTERNATIONAL SEARCH REPORT

International application No.
PCT/CA 02/01556

Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This International Search Report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. [X] Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:

Although claims 2-6 and 9-16 are directed to a diagnostic method practised on the human/animal body, the search has been carried out and based on the alleged effects of the compound/composition.

2. [X] Claims Nos.: 1-17 (partly)
because they relate to parts of the International Application that do not comply with the prescribed requirements to such an extent that no meaningful International Search can be carried out, specifically:

see FURTHER INFORMATION sheet PCT/ISA/210

3. [ ] Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. [ ] As all required additional search fees were timely paid by the applicant, this International Search Report covers all searchable claims.

2. [ ] As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.

3. [ ] As only some of the required additional search fees were timely paid by the applicant, this International Search Report covers only those claims for which fees were paid, specifically claims Nos.:

4. [ ] No required additional search fees were timely paid by the applicant. Consequently, this International Search Report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

[ ] The additional search fees were accompanied by the applicant's protest.

[ ] No protest accompanied the payment of additional search fees.

FURTHER INFORMATION CONTINUED FROM PCT/ISA/ 210

Continuation of Box I.2

Claims Nos.: 1-17 (partly)

Present claims 1-17 relate to a compound defined by reference to a desirable characteristic or property, namely "kallikrein binding molecule" or "an agent that binds kallikrein".

The claims cover all compounds having this characteristic or property, whereas the application provides support within the meaning of Article 6 PCT and/or disclosure within the meaning of Article 5 PCT for only a very limited number of such compounds. In the present case, the claims so lack support, and the application so lacks disclosure, that a meaningful search over the whole of the claimed scope is impossible. Independent of the above reasoning, the claims also lack clarity (Article 6 PCT). An attempt is made to define the compound by reference to a result to be achieved. Again, this lack of clarity in the present case is such as to render a meaningful search over the whole of the claimed scope impossible. Consequently, the search has been carried out for those parts of the claims which appear to be clear, supported and disclosed, namely those parts relating to antibodies to kallikreins.

The applicant's attention is drawn to the fact that claims, or parts of claims, relating to inventions in respect of which no international search report has been established need not be the subject of an international preliminary examination (Rule 66.1(e) PCT). The applicant is advised that the EPO policy when acting as an International Preliminary Examining Authority is normally not to carry out a preliminary examination on matter which has not been searched. This is the case irrespective of whether or not the claims are amended following receipt of the search report or during any Chapter II procedure.

INT[redacted]IONAL SEARCH REPORT

Information on patent family members

Int[redacted]ial Application No
PCT/CA 02/01556

Patent document cited in search report	Publication date	Patent family member(s)		Publication date
WO 0159158 A	16-08-2001	US	6294344 B1	25-09-2001
		AU	3491001 A	20-08-2001
		EP	1254267 A1	06-11-2002
		WO	0159158 A1	16-08-2001
		US	2002146708 A1	10-10-2002
		US	2002142317 A1	03-10-2002
WO 9811238 A	19-03-1998	AU	4480797 A	02-04-1998
		WO	9811238 A2	19-03-1998
US 6068830 A	30-05-2000	AU	7261694 A	13-02-1995
		WO	9502424 A1	26-01-1995
		US	6274118 B1	14-08-2001
WO 0235232 A	02-05-2002	AU	1370402 A	06-05-2002
		WO	0235232 A2	02-05-2002